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(54) Title: MULTIANALYTE MOLECULAR ANALYSIS

(57) Abstract: The present invention provides methods and apparatus for the application of a particle array in bioassay format to perform qualitative and/or quantitative molecular interaction analysis between two classes of molecules (an analyte and a binding agent). The methods and apparatus disclosed herein permit the determination of the presence or absence of association, the strength of association, and/or the rate of association and dissociation governing the binding interactions between the binding agents and the analyte molecules. The present invention is especially useful for performing multiplexed (parallel) assays for qualitative and/or quantitative analysis of binding interactions of a number of analyte molecules in a sample.

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MULTIANALYTE MOLECULAR ANALYSIS**FIELD OF THE INVENTION**

The present invention relates to multiplexed bioassays for analyzing binding interactions between analytes and binding agents, including methods for determining the affinity constants and kinetic properties associated with analyte-binding agent interactions.

BACKGROUND OF THE INVENTION

The imprinting of multiple binding agents such as antibodies and oligonucleotides on planar substrates in the form of spots or stripes facilitates the simultaneous monitoring of multiple analytes such as antigens and DNA in parallel ("multiplexed") binding assays. The miniaturization of this array format for increasing assay throughput and studying binding kinetics are described, for example, in R. Ekins, F. W. Chu, *Clin. Chem.* **37**, 955-967 (1991); E. M. Southern, U. Maskos, J. K. Elder, *Genomics* **13**, 1008-1017 (1992). In recent years, this approach has attracted substantial interest particularly in connection with performing extensive genetic analysis, as illustrated in G. Ramsay, *Nat. Biotechnol.* **16**, 40-44 (1998); P. Brown, D. Botstein, *Nat. Genet.* **21**, 33-37 (1999); D. Duggan, M. Bittner, Y. Chen, P. Meltzer, J. M. Trent, *Nat. Genet.* **21**, 10-14 (1999); R. Lipshutz, S. P. A. Fodor, T. R. Gingeras, D. J. Lockhart, *Nat. Genet.* **21**, 20-24 (1999).

The principal techniques of array fabrication reported to date include: refinements of the original "spotting" in the form of pin transfer or ink jet printing of small aliquots of probe solution onto various substrates, as illustrated in V. G. Cheung, et al., *Nat. Genet.* **21**, 15-19 (1999); sequential electrophoretic deposition of binding agents in individually
5 electrically addressable substrate regions, as illustrated in J. Cheng, et al., *Nat. Biotechnol.* , 541-546 (1998); and methods facilitating spatially resolved in-situ synthesis of oligonucleotides, as illustrated in U. Maskos, E. M. Southern, *Nucleic Acids Res.* **20**, 1679-1684 (1992); S. P. A. Fodor, et al., *Science* **251**, 767-773 (1991) or copolymerization of oligonucleotides, as illustrated in A. V. Vasiliskov, et al., *BioTechniques* **27**, 592-606
10 (1999). These techniques produce spatially encoded arrays in which the position within the array indicates the chemical identity of any constituent probe.

The reproducible fabrication of customized arrays by these techniques requires the control of microfluidics and/or photochemical manipulations of considerable complexity to
15 ensure consistent performance in quantitative assays. Microfluidic spotting to produce, in quantitatively reproducible fashion, deposited features of 100 μ m diameter involves dispensing of nanoliter aliquots with tight volume control, a task that exceeds the capabilities of currently available fluid handling methodologies. In addition, exposure of binding agents to air during the deposition process, typically several hours' in duration, has
20 uncontrollable impact on the molecular configuration and the accessibility of the binding agents in subsequent binding assays. In-situ array synthesis relies on a sequence of multiple masking and photochemical reaction steps which must be redesigned to accommodate any changes in array composition. Finally, assay performance must be assessed "in-situ" for each array subsequent to immobilization of binding agents, an aspect of array
25 manufacturing which raises difficult quality control and implementation issues.

SUMMARY OF THE INVENTION

The present invention provides methods and apparatus for the application of a particle array in bioassay format to perform qualitative and/or quantitative molecular interaction analysis between two classes of molecules (an analyte and a binding agent). The methods and apparatus disclosed herein permit the determination of the presence or
5 absence of association, the strength of association, and/or the rate of association and dissociation governing the binding interactions between the binding agents and the analyte molecules. The present invention is especially useful for performing multiplexed (parallel) assays for qualitative and/or quantitative analysis of binding interactions.

10

The terms “analyte” and “binding agent” refer to molecules involved in binding interactions. In one example, analyte and binding agent include DNA or RNA fragments (e.g., oligonucleotide), and binding of those fragments to their complementary sequences (hybridization) is analyzed. In another example, binding interactions between ligands and
15 receptors are analyzed. Examples of analytes and binding agents also include aptamers, peptides and proteins (e.g., antibodies), antigens, and small organic molecules.

The term “particles” refer to colloidal particles, including beaded polymer resins (“beads”).

20

The present invention also provides automated, on-demand fabrication of planar arrays composed of a selected mixture of chemically distinct beads (e.g., encoded beads) which are disposed on a substrate surface in accordance with a selected spatial configuration, as described above. In this approach, the beads are functionized to display
25 binding agents. For example, the binding agents may be attached to the beads, preferably by covalent bond. The subsequent quality control and performance evaluation are conducted off-line and are independent from the process of array assembly. The separation of steps such as bead encoding, functionalization and testing; substrate design, processing and evaluation; custom assembly of application-specific arrays; and on-line decoding of

arrays enable an otherwise elusive combination of flexibility, reliability and low cost by permitting systematic process control.

5 The methods disclosed herein permit rapid customization of DNA or protein arrays without the need for process redesign and avoid problems contributing to spot-to-spot as well as chip-to-chip variability. Furthermore, the bead array format permits chip-independent characterization of beads as well as optimization of assay conditions. In addition, multiple bead arrays can be formed simultaneously in discrete fluid compartments maintained on the same chip, permitting the concurrent processing of multiple samples.

10

BRIEF DESCRIPTION OF DRAWINGS

Other objects, features and advantages of the invention discussed in the above brief explanation will be more clearly understood when taken together with the following detailed description of an embodiment which will be understood as being illustrative only, and the accompanying drawings reflecting aspects of that embodiment, in which:

20 Fig. 1 is an illustration of process flow including the production of random encoded bead arrays and their use in multiplexed assays

Fig. 2 is an illustration of the functionalization of beads

Fig. 3 is an illustration of steps in chip design and wafer-scale production

Fig. 4 is an illustration of on-demand assembly of random encoded arrays

Fig. 5 is an illustration of palmtop microlab

25 Fig. 6 is a schematic illustration of assay and decoding images used in

READ process

Fig. 7 is a flow chart summarizing algorithms and steps in the analysis of images

Fig. 8 is an illustration of steps in the decomposition of assay images according to bead type by application of the image analysis algorithm summarized in Fig. 7.

Fig. 9 is an illustration of optically programmable array assembly of random encoded arrays

5 Fig. 10 is an illustration of an array composed of random encoded subarrays

Fig. 11 is an illustration of stations in an automated chip-scale bead array manufacturing and QC process

Fig. 12 is an illustration of quantitative binding curves for two cytokines

Fig. 13 is an illustration of array design for polymorphism analysis

10 Fig. 14 is a fluorescence micrograph of assay and decoding images recorded from one subarray shown in Fig. 13 in the course of polymorphism analysis

Fig. 15 is an illustration of assay results in the form of intensity histograms obtained from the analysis of assay images such as the one illustrated in Fig. 14.

15 Fig. 16 is an illustration of design of a "looped probe" for hybridization assays

Fig. 17A and 17B are fluorescence micrographs of assay and decoding images recorded in the course of the analysis of multiple cytokines

Fig. 18A and 18B are illustrations of numerical simulations of cross-correlations in receptor-ligand systems with multiple competing receptor-ligand interactions

20 Fig. 19 is an illustration of numerical simulations of receptor-ligand association and disassociation kinetics

Fig. 20 is an illustration of integrated sample capture using magnetic capture beads and array-based detection using READ

25 Fig. 21 is an illustration of multi-step assays using encoded magnetic beads to integrate gene-specific capture, on-bead reverse transcription and post-assay array assembly

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The fabrication of application-specific bead arrays may involve multiple processes in a multi-step sequence which may be automated using existing liquid handling technology and laboratory automation. The process of Random Encoded Array Detection (READ) includes the fabrication of custom bead arrays as well as the use of such arrays in bioassays, including assays involving multiplexed molecular interaction analysis of analyte and binding agent molecules, including DNA and protein analysis.

Fig. 1 provides a schematic overview of the functional components and process flow by which custom bead arrays may be prepared and used in performing multiplexed biomolecular analysis according to the present invention. The array is prepared by employing separate batch processes to produce application-specific substrates (e.g., chip at the wafer scale) and to produce beads that are chemically encoded and biologically functionalized (e.g., at the scale of $\sim 10^8$ beads/100 μ l of suspension). The beads subjected to respective quality control (QC) steps prior to array assembly, such as the determination of morphological and electrical characteristics. In addition, actual assays are performed on beads in suspension, before they are introduced to the substrate, to optimize assay conditions, generally with the objective to maximize assay sensitivity and specificity and to minimize bead-to-bead variations. For substrates, QC steps may include optical inspection, ellipsometry and electrical transport measurements.

20

Once the chemically encoded and biologically functionalized beads are combined with the substrate (e.g., chip), the Light-controlled Electrokinetic Assembly of Particles near Surfaces (LEAPS) may be used for rapid assembly of dense arrays on a designated area on the substrate within the same fluidic phase, avoiding problems contributing to spot-to-spot as well as chip-to-chip variability without the need for retooling or process redesign. Furthermore, the bead array format permits chip-independent characterization of beads as well as optimization of assay conditions. In addition, multiple bead arrays can be formed simultaneously in discrete fluid compartments maintained on the same chip. Once formed, these multiple bead arrays may be used for concurrent processing of multiple

samples. The integration of LEAPS with microfluidics produces a self-contained, miniaturized, optically programmable platform for parallel protein and DNA analysis. LEAPS refers to methods of moving particles suspended in the interface between an electrolyte solution and an electrode and is described in U.S. Patent Application Serial No.

- 5 09/171,550 (also PCT International Application No. PCT/US97/08159) entitled Light-controlled Electrokinetic Assembly of Particles near Surfaces, which is incorporated herein by reference in its entirety. Also incorporated herein by reference in its entirety is U.S. Patent Applications Serial No. 09/397,793 (also PCT International Application PCT/US00/25466), entitled "System and Method for Programmable Pattern Generation.

10

- In certain embodiments of the present invention, chemical encoding may be accomplished by staining beads with sets of optically distinguishable tags, such as those containing one or more fluorophore dyes spectrally distinguishable by excitation wavelength, emission wavelength, excited-state lifetime or emission intensity. The optically
- 15 distinguishable tags made be used to stain beads in specified ratios, as disclosed, for example, in Fulwyler, US 4,717,655 (Jan 5, 1988). Staining may also be accomplished by swelling of particles in accordance with methods known to those skilled in the art, [Molday, Dreyer, Rembaum & Yen, J. Mol Biol 64, 75-88 (1975); L. Bangs, "Uniform latex Particles, Seragen Diagnostics, 1984]. For example, up to twelve types of beads
- 20 were encoded by swelling and bulk staining with two colors, each individually in four intensity levels, and mixed in four nominal molar ratios. Combinatorial color codes for exterior and interior surfaces is disclosed in International Application No. PCT/US 98/10719, which is incorporated herein by reference in its entirety.

- 25 Beads are functionalized by binding agent molecules attached thereto, the molecule including DNA (oligonucleotides) or RNA fragments, peptides or proteins, aptamers and small organic molecules in accordance processes known in the art, e.g., with one of several coupling reactions of the known art (G. T. Hermanson, *Bioconjugate Techniques* (Academic Press, 1996); L. Illum, P. D. E. Jones, *Methods in Enzymology* 112, 67-84

(1985). In certain embodiments of the invention, the functionalized beads have binding agent molecules (e.g., DNA, RNA or protein) covalently bonded to the beads. Beads may be stored in a buffered bulk suspension until needed. Functionalization typically requires one-step or two-step reactions which may be performed in parallel using standard liquid handling robotics and a 96-well format to covalently attach any of a number of desirable functionalities to designated beads, as illustrated in Fig. 2. In a preferred embodiment, beads of core-shell architecture will be used, the shell composed in the form of a thin polymeric blocking layer whose preferred composition is selected; and functionalization performed in accordance with the targeted assay application, as known in the art. Samples may be drawn for automated QC measurements. Each batch of beads provides material for hundreds of thousands of chips so that chip-to-chip variations are minimized.

Substrates (e.g., chips) used in the present invention may be patterned in accordance with the interfacial patterning methods of LEAPS by, e.g., patterned growth of oxide or other dielectric materials to create a desired configuration of impedance gradients in the presence of an applied AC electric field. Patterns may be designed so as to produce a desired configuration of AC field-induced fluid flow and corresponding particle transport. Substrates may be patterned on a wafer scale by invoking semiconductor processing technology, as illustrated in Fig. 3. In addition, substrates may be compartmentalized by depositing a thin film of a UV-patternable, optically transparent polymer to affix to the substrate a desired layout of fluidic conduits and compartments to confine fluid in one or several discrete compartments, thereby accommodating multiple samples on a given substrate.

In certain embodiments of the invention, the bead array is prepared by providing a first planar electrode that is in substantially parallel to a second planar electrode ("sandwich" configuration) with the two electrodes being separated by a gap and containing an electrolyte solution. The surface or the interior of the second planar electrode is patterned with the interfacial patterning method. Encoded and functionalized

beads are introduced into the gap. When an AC voltage is applied to the gap, the beads form a random encoded array on the second electrode (e.g., "chip"). And, also using LEAPS, an array of beads may be formed on a light-sensitive electrode ("chip").

5 Preferably, the sandwich configuration described above is also used with a planar light sensitive electrode and another planar electrode. Once again, the two electrodes are separated by the a gap and contain an electrolyte solution. The functionalized and encoded beads are introduced into the gap. Upon application of an AC voltage in combination with a light, the beads form an array on the light-sensitive electrode.

10 In certain embodiments, the application-specific bead arrays useful in the present invention may be produced by picking aliquots of designated encoded beads from individual reservoirs in accordance with the specified array composition and "pooled"; aliquots of pooled suspension are dispensed onto selected substrate (e.g., chips) in a manner preventing the initial fusion of aliquots. Aliquots form a multiplicity of planar random subarrays of encoded beads, 15 each subarray representing beads drawn from a distinct pool and the physical array layout uniquely corresponding to the identity of aliquots drawn from pooled bead populations.

Planar arrays or assemblies of encoded on a substrate which are chemically or physically encoded may be used. To this, spatial encoding may also be added to increase the 20 number of assays that may be conducted. Spatial encoding, for example, can be accomplished within a single fluid phase in the course of array assembly by invoking Light-controlled Electrokinetic Assembly of Particles near Surfaces (LEAPS) to assemble planar bead arrays in any desired configuration in response to alternating electric fields and/or in accordance with patterns of light projected onto the substrate. LEAPS creates lateral gradients in the 25 impedance of the interface between silicon chip and solution to modulate the electrohydrodynamic forces that mediate array assembly. Electrical requirements are modest: low AC voltages of typically less than $10V_{pp}$ are applied across a fluid gap of typically $100\text{ }\mu\text{m}$ between two planar electrodes. This assembly process is rapid and it is optically programmable: arrays containing thousands of beads are formed within seconds under electric

field. The formation of multiple subarrays, can also occur in multiple fluid phases maintained on a compartmentalized chip surface.

The multiplexed assays of the present invention may also be performed using beads
5 encoded beads that are assembled, but not in an array, on the substrate surface. For example, by spotting bead suspensions into multiple regions of the substrate and allowing beads to settle under gravity, assemblies of beads can be formed on the substrate. In contrast to the bead arrays formed by LEAPS, these assemblies generally assume low-density, disorder configurations. However, the combination of spatial and color encoding attained by spotting
10 mixtures of chemically encoded beads into a multiplicity of discrete positions on the substrate still provides a degree of multiplexing that is sufficient for certain biological assays.

Binding interaction between the binding agent on those beads and an analyte may be performed either before or after the encoded array is assembled on the substrate. For
15 example, the bead array may be formed after the assay, subsequent to which an assay image and a decoding image may be taken of the array. Alternatively, the beads may be assembled in an array and immobilized by physical or chemical means to produce random encoded arrays, e.g., with the appearance of the array shown in Fig.10. The arrays may be immobilized, for example, by application of a DC voltage to produce random encoded arrays with the
20 appearance of the array shown in Fig. 10. The DC voltage, set to typically 5-7 V (for beads in the range of 2-6 μm and for a gap size of 100-150 μm) and applied for < 30s in "reverse bias" configuration so that an n-doped silicon substrate would form the anode, causes the array to be compressed to an extent facilitating contact between adjacent beads within the array and simultaneously causes beads to be moved toward the region of high electric field in immediate
25 proximity of the electrode surface. Once in sufficiently close proximity, beads are anchored by van der Waals forces mediating physical adsorption. This adsorption process is facilitated by providing on the bead surface a population of "tethers" extending from the bead surface; polylysine and streptavidin have been used for this purpose.

In certain embodiments, the particle arrays may be immobilized by chemical means, e.g., by forming a composite gel-particle film. In one exemplary method for forming such gel-composite particle films, a suspension of microparticles is provided which also contain all ingredients for subsequent in-situ gel formation, namely monomer, crosslinker and initiator. The particles are assembled into a planar assembly on a substrate by application of LEAPS, e.g., AC voltages of 1-20 V_{pp} in a frequency range from 100's of hertz to several kilohertz are applied between the electrodes across the fluid gap. Following array assembly, and in the presence of the applied AC voltage, polymerization of the fluid phase is triggered by thermally heating the cell ~ 40-45°C using an IR lamp or photometrically using a mercury lamp source, to effectively entrap the particle array within a gel. Gels may be composed of a mixture of acrylamide and bisacrylamide of varying monomer concentrations from 20% to 5% (acrylamide : bisacrylamide = 37.5 : 1, molar ratio), or any other low viscosity water soluble monomer or monomer mixture may be used as well. Chemically immobilized functionalized microparticle arrays prepared by this process may be used for a variety of bioassays, e.g., ligand receptor binding assays.

In one example, thermal hydrogels are formed using azodiisobutyramidine dihydrochloride as a thermal initiator at a low concentration ensuring that the overall ionic strength of the polymerization mixture falls in the range of ~ 0.1mM to 1.0mM. The initiator used for the UV polymerization is Irgacure 2959® (2-Hydroxy-4'-hydroxyethoxy-2-methylpropiophenone, Ciba Geigy, Tarrytown, NY). The initiator is added to the monomer to give a 1.5 % by weight solution.

In certain embodiments, the particle arrays may be immobilized by mechanical means. For example, an array of microwells may be produced by standard semiconductor processing methods in the low impedance regions of the silicon substrate. The particle arrays may be formed using such structures by, e.g., utilizing LEAPS mediated hydrodynamic and ponderomotive forces are utilized to transport and accumulate particles on the hole arrays. The A.C. field is then switched off and particles are trapped into

microwells and thus mechanically confined. Excess beads are removed leaving behind a geometrically ordered random bead array on the substrate surface.

When the bead array is immobilized before the assay, the array functions as a two-
5 dimensional affinity matrix which displays receptors or binding agents (e.g.,
oligonucleotides, cDNA, aptamers, antibodies or other proteins) to capture analytes or
ligands (DNA, proteins or other small cognate ligands) from a solution that is brought in
contact with the array. The bead array platform may be used to perform multiplexed
molecular analysis, such as, e.g., genotyping, gene expression profiling, profiling of
10 circulation protein levels and multiplexed kinetic studies.

Substrates (e.g., chips) can be placed in one or more enclosed compartment,
permitting samples and reagents to be transported in and out of the compartments through
fluidic interconnection. On-chip immunoassays for cytokines, e.g., interleukin (IL-6) may
15 be performed in this format. Serum sample and fluorescent labeled secondary antibodies are
introduced to the reaction chamber sequentially and allowed to react with beads
immobilized on the chip. Fig. 5 illustrates a design of a reaction chamber which may be
used in the multiplexed assays according to the present invention. Reactions can also be
performed in an open compartment format similar to microtiter plates. Reagents may be
20 pipetted on top of the chip by robotic liquid handling equipment, and multiple samples may
be processed simultaneously. Such a format accommodates standard sample processing and
liquid handling for existing microtiter plate format and integrates sample processing and
array detection.

25

In certain embodiments, the presence of the analyte-binding agent interactions are
associated with changes in the optical signatures of beads involved in the interactions and
these optical changes detected and analyzed. The identities of the binding agents involved
in the interactions are determined by detecting the chemically or physically distinguishable

characteristic associated with those beads. Preferably, chemically distinguishable characteristics include chemical molecules including fluorephore dyes, chromophores and other chemical molecules that are used for purposes of detection in binding assays.

5 The detection of the chemically or physically distinguishable characteristic and the detecting of the optical signature changes associated with the binding interactions may be performed while the particles are assembled in a planar array on a substrate, e.g., by taking an assay and a decoding image of the array and comparing the two, e.g., comparing of the assay and the decoding image comprises use of optical microscopy apparatus including an
10 imaging detector and computerized image capture and analysis apparatus. The decoding image may be taken to determine the chemically and/or physically distinguishable characteristic that uniquely identifies the binding agent displayed on the bead surface, e.g., determining the identity of the binding agents on each particle in the array by the distinguishable characteristic. The assay image of the array is taken to detect the optical
15 signature of the binding agent and the analyte complex. In certain embodiments, fluorescent tags (fluorophore dyes) may be attached to the analytes such that when the analytes are bound to the beads, the fluorescent intensities change, thus providing changes in the optical signatures of the beads. In certain embodiments, the decoding image is taken after the beads are assembled in an array and immobilized and before taking the assay
20 image, preferably before contacting the binding agents on the beads with an analyte. In certain other examples, the binding interactions occur while the beads are in solution, and assembled into an array afterwards and the decoding and assay images are obtained. The identity of the binding agent of the binding agent-analyte complex is carried out by comparing the decoding image with the assay image.

25

 In preferred embodiments, images analysis algorithms that are useful in analyzing the data obtained from the decoding and the assay images. These algorithm may be used to obtain quantitative data for each bead within an array. As summarized in Fig. 7, the analysis software automatically locates bead centers using a bright-field image of the array as a

template, groups beads according to type, assigns quantitative intensities to individual beads, rejects "blemishes" such as those produced by "matrix" materials of irregular shape in serum samples, analyzes background intensity statistics and evaluates the background-corrected mean intensities for all bead types along with the corresponding variances.

5

The methods of the present invention may be used for determining the association and the dissociation constants e.g., by introducing the analyte in a time-dependent manner and analyzing the binding as a function of time, or by washing away the bound analyte in a time-dependent manner and also analyzing the binding as a function of time.

10

The methods of the present invention may be used for determining the affinity constants of analyte-binding agent interactions, for determining the number of analyte-binding agent complexes formed

15

The present invention also provides methods for determining the concentration of an analyte in a biological sample.

20

The methods of the present invention may also be used to determining elements of a co-affinity matrix of a given analyte against a panel of binding agents. In one example, the extent of the interaction between the analyte and the binding agents in a panel in competitive, multiconstituent equilibrium reaction may be determined. Determination of co-affinity constants provides useful applications, as described below.

25

The successful rate of transplantation for several types of organs directly relates to compatibility of Human Leukocyte Antigen (HLA) between donor and recipient. Serological testing of the recipients for the Panel Reactive Antibodies (PRA) is one of the crucial steps to avoid possible rejections. Cross-reaction in PRA testing is a very common phenomenon due to similarity of some HLA antigen structures and the nature of development of these antibodies. In fact, HLA antigens can be organized into groups based

on apparent serological cross-reactivity between the groups. These groups are termed Cross-Reactive-Groups (CREGs). In current clinical setting, antibodies from a patient are tested against different antigens in individual reactions. Although a reactive pattern of the antibodies can be generated combining the results from different reactions, the competitive
5 nature of interactions between different antibodies and antigens is not reflected in such a pattern. In other cases, several antigens are mixed together for a binding assay. Lack of identification of each antigen in the system prevents generation of a binding profile. The result is only the averaged signal from several antigens. In the bead array system, a panel of different antigens is presented to the antibody analytes in a competitive binding
10 environment, and each antigen can be identified through its association with different types of beads. Thus, binding intensity on each antigen in the competitive reactions can be extracted in a single assay. This co-affinity matrix system will provide binding profiles for the CREGs and greatly advance the understanding of the nature of the reaction and improve the accuracy for the related clinical decisions. For example, a N-antibody and M-
15 antigen system provides a matrix of $N \times M$ of possible reactions. It is possible to determine K_{nm} , the affinity constant governing the interaction between the n th antibody against the m th antigen, where $m = 1, 2, \dots, M$, and $n = 1, 2, \dots, N$. For applications where absolute co-affinity constants are not needed, binding profile will be generated for various antibodies in accordance with the methods of the present invention and results from a patient sample can
20 be matched to these profiles or combination of these profiles.

Co-affinity matrix may also be used to characterize the analyte. For example, combination of the coefficients of the co-affinity matrix and known concentrations of analyte and binding agents participating in the formation of analyte-binding agent
25 complexes serves to define a competitive binding interaction descriptor, e.g., The molecular interaction parameter,

$$P_n(R_m) = \frac{K_{mn}[L_n]}{\sum_j K_{mj}[L_j]}$$

provides a characterization of the molecular interaction between a binding agent, R_m , and
 5 an analyte, L_n , in the presence of analytes $\{L_j ; 1 \leq j \leq N\}$, all of which exhibit a finite
 affinity, K_{mj} , for that binding agent. That is, P_n , $0 \leq P_n \leq 1$, represents a normalized
 specificity of binding agent R_m for analyte L_n in a multiconstituent competitive reaction and
 serves as a robust characterization of that binding agent based on co-affinities displayed in a
 multiconstituent competitive reaction. See also P.H. von Hippel et al., Proc. Natl. Acad.
 10 Sci. USA 83, 1603 (1986), incorporated herein by reference.

The pattern of binding interaction of an analyte against a panel of binding agents may
 be used to characterize the analyte and compare it with other molecules. In addition, by
 generating the co-affinity matrix of an analyte using a reference panel of binding agents, such
 15 affinity may be used to determine if a sample later introduced to the panel of binding agents
 contains an impurity by observing the deviation in the binding pattern.

The present invention also provides use of superparamagnetic particles ("magnetic
 particles") as described in U.S. Patent No. 5, 759,820 and European Patent No.
 20 83901406.5 (Sintef), which may then be used in integrated the sample preparation step with
 the assay step involving encoded bead arrays. Both of these references are incorporated
 herein by reference.

Superparamagnetic particles may be encoded with a chemically or physically
 25 distinguishable characteristic (e.g., fluorescent tag) and used performing bioassays of the

present invention. In certain embodiments, the particles are assembled using LEAPS, as with non-magnetic encoded beads. The encoded also be used in array generation, and assayed. The present invention also includes the formation of a planar array of encoded and functionalized superparamagnetic particles on a substrate by application of magnetic
5 field to said particles.

Several methods for the synthesis of monodisperse superparamagnetic microspheres are known in the art. G. Helgesen et al., Phys. Rev. Lett. 61, 1736 (1988), for example, disclosed a method which utilizes porous and highly cross-linked polystyrene core particles
10 whose interior surfaces are first nitrated, following which iron oxides are precipitated throughout the particle to produce a paramagnetic core. Following completion of this step, the particles are coated with functional polymers to provide a reactive shell. US patent 5,395,688 to Wang et al. describes a process for producing magnetically responsive fluorescent polymer particles composed of a fluorescent polymer core particle that is evenly
15 coated with a layer of magnetically responsive metal oxide. The method utilizes preformed fluorescent polymeric core particles which are mixed with an emulsion of styrene and magnetic metal oxide in water and polymerized. A two step reactive process such as this suffers from the drawback of possible inhibition of polymerization by the fluorescent dye or conversely bleaching of the fluorescence by the shell polymerization process.

20

The method also provides a novel process for making color encoded magnetic beads, a simple and flexible one-step process to introduce into preformed polymeric microparticles a well controlled amount of magnetic nanoparticles, prepared in accordance with the procedure described below, along with well controlled quantities of one or more
25 fluorescent dyes. In an embodiment of the present invention, the quantity of the magnetic nanoparticles. is controlled to produce magnetic particles that form an array on a substrate upon application of magnetic field to said particles. This process involves swelling the polymer particles in an organic solvent containing dyes and magnetic nanoparticles and therefore applies to any polymer particle which can be subjected to standard swelling

procedures such as those disclosed in the prior art of fluorescent staining of microparticles. Unlike encoding methods in which the magnetic material and the fluorescent dyes are each located to different areas of the (core/shell) of the magnetic particle, uniform swelling of particles ensures the distribution of magnetic particles throughout the interior volume. This process also permits the quantitative control of the nanoparticle as well as dye content over a wide range, thereby permitting the tailoring of the particles' magnetic susceptibility as well as fluorescence intensities. An additional method of the present invention to control the magnetic properties of the host particles, other than to control loading, is to tune the size of the magnetic nanoparticles by adjusting the water content of the micellar synthesis reaction (see below).

Physical or chemical coupling of biomolecules possible on the particle surface utilizing preexisting functional groups. Leaching out of magnetic nanoparticles is readily eliminated by growing a further polymeric shell on the particle.

15

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes and are not to be construed as limiting this invention in any manner.

20 **EXAMPLES**

Example 1: Optically programmable array formation

As illustrated in Fig. 9, LEAPS serves to simultaneously assemble multiple random encoded subarrays and to "drag-and-drop" these subarrays into separate, but proximate locations on the chip within a common, enclosed liquid environment. Two sets of beads (2.8 μm Oligo-(dT)₂₅, Dynal, Oslo, Norway), dispensed from separate reservoirs A and B, were simultaneously assembled into distinct subarrays within the same fluid; sub-arrays were then simultaneously placed into desired destinations as directed by spatially varying illumination

profiles which were generated and projected onto the substrate by a PC-programmable illumination pattern generator (described in U.S. Serial No. 09/397,793, filed September 17, 1999, which is incorporated herein by reference in its entirety). This drag-and-drop operation reduced the separation between the two sub-arrays from approximately 250 μm to 20 μm .

- 5 Beads were moved at 5 V_{pp} at a frequency of 2 kHz; total power projected onto the substrate surface was ~ 5 mW. The combination of chemical and spatial encoding permits a given set of chemical bead markers to be used multiple times and reduces the demands placed on either encoding dimension while facilitating the realization of large coding capacities.

10 **Example 2: Array formation on patterned surface**

- Illustrated in Fig. 10 is an array of encoded beads assembled on a patterned silicon chip using an AC voltage of 1-2 V_{pp} and a frequency of 100-150 Hz, applied across a 100 μm electrode gap filled with an aqueous bead suspension; a thermal oxide (~ 1000 Å) on the substrate was patterned by etching the oxide to a thickness of 50-100 Å in a set of square features ($\sim 30 \times 30 \mu\text{m}^2$) on 130 μm centers; arrays of similar layout also can be produced in response to suitable illumination patterns. Each sub-array shown here contains approximately 80 beads coupled with anti-cytokine monoclonal antibodies. Carboxylate-modified polystyrene beads of 5.5 μm diameter (Bangs Laboratory, Fishers, IN) were stained with a combination of two types of fluorescent dyes and were then functionalized with anti-cytokine-mAb. The assembly process ensures collection of all beads at the substrate surface. Bead encoding was as follows: IL-2 (Bright Red); IL-4 (Dim Red); IL-6 (Bright Green); M-CSF (Dim Green) and TNF- α (Yellow).

25 **Example 3: Formation of arrays of magnetic particles**

Colloidal particles exhibiting a finite diamagnetic susceptibility, when disposed on a planar substrate can be assembled into ordered arrays in response to increasing magnetic fields. Commercially available superparamagnetic particles (Dynal, Oslo, NO), dispersed from a fluid suspension onto the planar surface of the lower of two parallel bounding surfaces of a

fluid cell ("sandwich" geometry), when exposed to a homogeneous axial magnetic field (oriented normal to the substrate plane), will form ordered assemblies. As a function of increasing magnetic field strength, and for given diamagnetic susceptibility of the particles as controlled by the manufacturing process known to the art, ordered planar assemblies and linear strings of beads oriented normal to the substrate can be formed. Permanent magnets can be designed so as to produce the field strength required to realize the desired configuration of the assembly. Requisite magnetic field configurations can be produced by an electromagnet in solenoid or Helmholtz configuration known to the art; the substrate can be introduced into the magnet bore or can be placed in immediate proximity to the coil(s) outside of the bore so as to ensure the orientation of the field substantially normal to the substrate plane. Spatially modulated magnetic fields can be produced by patterning the substrate with permalloy using methods known to the art.

Example 4: Formation of random bead assemblies

Aliquots of solution containing suspended beads were placed onto several distinct positions on a planer substrate of silicon capped with a thin silicon oxide layer (other substrates may be used here). Beads were allowed to settle under gravity to form random assemblies. To delineate discrete positions on the substrate, one of the following two methods were used. According to the first method, a silicon gasket (of 250 μm thickness), displaying a grid of multiple round holes of 1 mm or 2 mm diameter (Grace Bio-labs, Bend, Oregon) is placed on the hydrophillic surface to define microwells (of 0.25 to 0.5 μl volume) for multiple discrete samples of bead suspension. According to the second method, small aliquots of fluid containing beads (0.2 μl to 0.5 μl in volume) are directly placed onto a hydrophillic surface in one or more designated areas so as to ensure formation of discrete droplets; spacers are not needed in this case. As solvent evaporates (at room temperature or, for rapid drying, at elevated temperature (about 60 C), beads are left in random positions on the substrate. DNA polymorphism reactions have been tested in assemblies formed in both manners. Optionally, beads settling under gravity may be immobilized by chemical capture layers provided on the

substrate. An application of random bead assemblies to determine affinity constants in a multiplexed format is described in Example 6.

Example 5: An automated chip-scale array manufacturing process

5 As illustrated in Fig. 11, the process involves liquid handling and pipetting of beads onto chips mounted in single-chip cartridges or multi-chip cartridges. Bead arrays are formed using methods such as those in Examples 1, 2 or 3., followed by array immobilization and decoding. The resulting decoding images are stored for later use along with an optional chip ID ("bar code").

10

Example 6: Determination of affinity constants by post-assay analysis of bead assemblies.

Quantitative binding curves for the cytokines TNF- α and IL-6. Binding curves were generated by performing sandwich immunoassays using chemically encoded beads in
15 suspension, said suspensions being confined to one or more reaction compartments delineated on-chip, or in one or more reaction compartments off chip. By completing the reaction with beads maintained in suspension, assay kinetics similar to homogeneous assays can be attained. Following completion of the binding reaction, beads were assembled on chip to permit multiplexed quantitative image analysis. Random assemblies prepared according to Example
20 4 or ordered bead arrays prepared according to Example 1 or 2 may be used. An advantage of ordered, dense assemblies produced by the methods of Examples 1 or 2 is the higher spatial density and higher assay throughput attained by processing a greater number of beads.

As an illustration, Fig. 12 displays quantitative binding curves for TNF- α and
25 IL-6, obtained from randomly dispersed beads. A commercial-grade 8-bit video-CCD camera (Cohu, San Diego, CA) was used in a mode permitting multi-frame integration. The range of concentrations of antigen used in the two assays was 700 fM to 50 pM for TNF- α and 2 pM to 50 pM for IL-6. At each concentration, the number of molecules bound per bead was estimated by comparison with calibration beads coated with known quantities of Cy5.5-labeled

BSA per bead; requisite adjustments were made to account for differences in fluorescence quantum efficiency between labeled secondary antibodies and BSA.

This format of analysis permits the determination of the affinity constant, K_A
 $= [LR]/([R_0-LR][L])$, where, in accordance with the law of mass action, $[LR]$ denotes the
 5 number of receptor-ligand pairs per bead and $[L]$ denotes the solution concentration of ligand.
 By specifying the number of beads per ml, n_B , and specifying a value for $[R_0]$ in terms of the
 number of receptors per bead, theoretical binding curves, computed for given K_A , are
 compared to a plot of the number of bound molecules per bead as a function of bulk ligand
 concentration. The absolute number of ligands bound per bead may be determined for given
 10 bulk concentration by measuring the mean fluorescence intensity per bead and referencing this
 to the fluorescence intensity recorded from calibration beads included in the array.

The estimated number of molecules bound per bead is compared to theoretical
 binding curves derived from the law of mass action. The three curves shown correspond to
 values of the affinity constant, K_A , of 10^{11} /molar, 10^{10} /molar and 10^9 /molar, respectively. The
 15 initial number of antibodies per bead, R_0 , equals 2×10^5 /bead and $n_B = 10^5$ /ml. Each data point
 represents the average of three replicates, with an assay-to-assay variation of $< 45\%$. Setting
 the assay sensitivity to correspond to that level of fluorescence which yields a signal-to-noise
 level of unity in the assay images, the sensitivity of the cytokine assays characterized in Fig. 12
 is set at $\sim 2,000$ bound ligands/bead, corresponding to respective detected concentrations of
 20 700 fM for TNF- α and 2 pM for IL-6.

While commercial ELISA kits use enzymatic amplification to enhance
 sensitivity, at the expense of additional complexity relating to assay conditions and controls,
 our bead array assay format, even without enzymatic amplification, our on-chip assay format
 permits monitoring of cytokines at circulating levels (Normal TNF- α level in serum is 50-280
 25 fM and normal IL-6 level in serum is 0-750 fM.
www.apbiotech.com/technical/technical_index.html), providing a dynamic range which
 approaches that of standard, i.e. amplified single-analyte ELISA assays (Assay kits of R & D
 Systems and Amersham (not the recent High-Sensitivity assays). Further improvements at
 hardware and software levels are possible.

Example 7: Genotyping by Polymorphism Analysis

To illustrate the application of the present invention to the implementation of genotyping, Fig. 13 shows the design of the assay in which five pairs of 20-mer binding agents corresponding to four polymorphic regions of a gene were coupled to color-encoded beads. The pairs of binding agents $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$ each display a single nucleotide difference in their respective sequences; the pair $\delta 3$, $\delta 4$ displays a difference of three nucleotides, the binding agents in the set $\gamma 1$, $\gamma 3$, $\gamma 3$, $\gamma 4$ display small insertions and deletions. The ten binding agents were divided into two subgroups of five which were incorporated into two subarrays. In this example, there are several hundred beads for each type. Following bead immobilization, an on-chip hybridization reaction was performed in TMAC buffer (2.25 M tetramethylammonium chloride, 37 mM Tris pH 8.0, 3 mM EDTA pH 8.0, and 0.15% SDS) at 55°C for 30 min. The analyte is a 254-base PCR fragment produced from a patient sample and fluorescently labeled at the 5'-prime end with BODIPY 630/650 (Molecular Probes, Eugene, OR). Image acquisition was performed after replacing the assay buffer with fresh TMAC buffer.

Fig. 14 shows decoding and assay images for one subarray. Each bead shown in the assay image obtained after hybridization is analyzed to determine fluorescence intensity and bead type; as with the cytokine assay, the latter operation compares assay and decoding images using a template matching algorithm. Fig. 15 displays the resulting intensity histograms for each bead type: in these histogram plots, the horizontal axis refers to relative signal intensity from 0 to 1 and the vertical axes refer to bead numbers. The histograms show that most of the beads displaying probe $\alpha 1$ bind no analyte while most of the beads displaying probe $\alpha 2$ exhibit significant binding; the mean signal level of $\alpha 2$ -beads exceeds that of $\alpha 2$ -beads by a factor of ~ 3.2 , indicating that analyte contains DNA sequences complementary to $\alpha 2$ but not $\alpha 1$. For the

patient sample presented here, the histogram indicates a genotype of the analyte DNA characterized by complementarity to binding agents $\alpha 2$, $\beta 2$, $\gamma 3$, $\gamma 4$ and $\delta 4$ in the polymorphic region of the gene.

5 **Example 8: Gene Expression Analysis: cDNA Fragments**

The method of the present invention has been used to fabricate arrays composed of beads displaying oligonucleotides as well as DNA fragments (e.g., up to ~1,000 bases in length). Strands were biotinylated at multiple positions by nick-translation and were attached to streptavidin-functionalized beads (M-280, Dynal, Oslo, NO). Arrays were formed
10 using an AC voltage of 800 Hz at 10V_{pp}.

Example 9: Looped probe design for universal labeling

A looped probe design in Fig. 16 takes advantage of fluorescence energy transfer to obviate the need for labeled target. As with the molecular beacon design (S. Tyagi,
15 D. P. Bratu. F. R. Kramer, *Nature Biotech.* 16, 49-53 (1998)), the probe in Fig. 16 assumes two different states of fluorescence in the closed loop and open loop configurations, but in contrast to the molecular beacon contains a portion of its binding motif within the stem structure to permit molecular control of stringency in competitive hybridization assays.

20 **Example 10: Quantitative Multiplexed Profiling of Cytokine Expression**

Fig. 17 displays a pair of assay and decoding images recorded from a single random array in a multiplexed sandwich immunoassay. An array containing five distinct types of beads, each displaying a monoclonal anti-cytokine antibody (mAb), was exposed to a sample solution (such as serum) containing two cytokine antigens (Ag). Subsequent addition
25 of Cy5.5-labeled secondary antibodies (pAb*) results in the formation of ternary complexes, mAb-Ag-pAb*. The on-chip immunoassay was performed by adding 300 μ l of sample with 7 nM cytokines in assay buffer (20 mM NaPi pH 7.4, 150 mM NaCl, 10 mg/ml BSA) to the bead array immobilized on the chip, and allowing the reaction to proceed at 37°C for one hour. The buffer was replaced by adding 12 nM solution of labeled secondary antibodies in assay

buffer. After one hour of incubation at 37°C, fresh buffer was added on top of the chip and image acquisition was performed. Antibodies and antigens used in the assays were obtained from R&D Systems (Minneapolis, MN); the secondary antibody was labeled with Cy5.5 using a standard kit (Amersham Pharmacia Biotech, Piscataway, NJ).

5 The decoding image Fig. 17B shows five types of beads in a false-color display with the same encoding pattern as that of Fig. 10. All beads are of the same size (5.5 μm diameter); the apparent difference in the size of beads of different types in the decoding image is an artifact reflecting different internal bead staining levels and "blooming" during CCD recording of the decoding image. Comparison (using the image analysis methods disclosed
10 herein) of the decoding image with the assay image in Fig. 14A reveals that active beads, of yellow and bright green types, captured TNF- α and IL-6, respectively. This assay protocol has been extended to the following set of twelve cytokines: IL-1 α , IL-1 β , IL-2, IL-4, IL-6, TGF- β 1, IL-12, EGF, GM-CSF, M-CSF, MCP-1 and TNF- α . The on-chip immunoassay requires no additional washing other than changing reagent solutions between assay steps. Comparison
15 between assay and decoding images shows that two different cytokines were present in the sample, namely IL-6 and TNF- α . The pre-formed arrays described in this example also permit the determination of affinity constants in a manner analogous to the analysis described in Example 6.

20 **Example 11: Aptamers for Protein Profiling**

Aptamers may be selected from large combinatorial libraries for their high binding affinities to serum proteins (L. Gold, B. Polisky, O. Uhlenbeck, M. Yarus, *Annu. Rev. Biochem.* 64: 763-797. (1995)). Random encoded arrays of aptamer-functionalized beads would serve to monitor levels of serum proteins; correlations in binding patterns on the array
25 (see also Example 10) may serve as a phenotype of disease states.

Example 12: Mixed DNA -Protein Arrays

Of significant interest to genomic functional analysis is the fact that the method of the present invention accommodates protein and DNA arrays without change in array

manufacturing methodology. Specifically, mixed arrays composed of beads displaying DNA and corresponding proteins can be used to analyze the gene and gene product within the same fluid sample.

This has been demonstrated for a combination of immunoassay and DNA hybridization. For example, a mixed array composed of beads functionalized with anti-cytokine monoclonal antibodies (mAb) and with oligonucleotides was produced. Two sequential assays were performed on this single chip. First, an immunoassay was performed in accordance with the protocol described in Example 10. Following completion of the on-chip immunoassay, image is acquired and the DNA analyte was added to the hybridization buffer (2x SSC, 1x Denhardt's) at a final concentration of 20 nM and allowed to react at 37°C for 1 hr. Fresh hybridization buffer was added to the chip and image acquisition was performed to record of the additional hybridization assay.

Example 13. Affinity Fingerprinting

The analysis of receptor-ligand interactions relevant to prior art methods assumes ideal specificity. That is, only the ideal situation is considered of a single ligand present in solution reacting with its matching receptor and vice versa. However, in most multiple assay systems, a considerable level of cross-reactivity may exist. That is, any single ligand may associate with several receptors, while any single type of receptor may have a finite affinity towards more than one ligand.

The present invention includes a model that is developed to analyze multiplexed READ assays for such a system under the following assumptions: each of these reversible reactions is characterized by its own affinity constant; no reaction occurs between the bulk species; there is no interaction between the complexes formed on the surface. These assumptions can be relaxed, at the expense of increasing the complexity of modeling, by accounting for reactions in the bulk and between the surface species. The standard reaction-diffusion equation for single receptor-ligand pair formation [R. W. Glaser, Anal. Biochem. 213, 152-161 (1993)], is generalized to allow for multiple reactions at each bead surface:

$$\frac{\partial [L_i \cdot R_j]}{\partial t} = k_{on,ij} [L_i] \left([R_{j,0}] - \sum_{n,m} [L_m \cdot R_n] \right) - k_{off,ij} [L_i \cdot R_j] \quad \forall i, j, L_i \equiv L_i(t, x, 0) \quad (2)$$

The first term on the right of Eq. (1) describes the association of ligands and receptors into complexes and involves of concentration of free sites on the surface. The second term describes the disassociation of complexes by way of release of ligands, thereby freeing up receptor sites for further reaction. Since a maximum of (i×j) bimolecular complexes can form, there could be as many boundary conditions generated from the above equation. For the equilibrium case, the left hand-side of Eq. (1) is set to zero, and the matrix of coaffinities, $[K_{ij}] = k_{on,ij}/k_{off,ij}$, can then be defined to accommodate cross-reactivities between multiple species in the bulk and on the surface. In a batch reactor under equilibrium conditions, we may solve the system of differential equations to obtain the number of molecules of each ligand bound on beads of each type.

L_1	Ligand concentration	10 pM
L_2	Ligand concentration	100 pM
R_{01}	Initial receptor concentration	1×10^4 /bead
R_{02}	Initial receptor concentration	1×10^4 /bead
n_{B1}	Bead number density	1×10^4 /ml
n_{B2}	Bead number density	1×10^4 /ml
$[K]$	Coaffinity matrix	$[1 \times 10^{11} \ 1 \times 10^9$ $1 \times 10^8 \ 1 \times 10^{11}]$ l/mole

20

As an illustrative example, the ligand distribution has been calculated (from the model in Eq (1)) for a reference set of two ligands and two types of receptors immobilized on

two different sets of beads. The coaffinity matrix is assumed known for each ligand-receptor combination in the reference set; to investigate the detection of a third ligand, it is assumed here that diagonal elements of the 2x2 matrix, $[K_{ij}]$, are large compared to off-diagonal elements. The presence of a third ligand in the reactor alongside the two original ligands
 5 perturbs the equilibria between the various complexes and the reactants in the reference system, and for ligand molecules tagged with fluorescent labels, the intensity observed from the perturbed system differs from that observed in the reference case.

Fig. 18A shows the reference case in which the concentrations and coaffinity matrix were set to the values shown in the accompanying table; the bead intensity was defined
 10 on a linear scale of 0 - 255, the latter representing the intensity of the brightest beads. Fig. 18A shows the contribution of each ligand to the bead intensity. Due to the lower concentration of L_1 , the intensity of Bead 1 is less compared to Bead 2, cross-reactivities are essentially undetectable.

Next, the system was perturbed with a third ligand, taking the concentration
 15 L_3 to be 1 pM and assuming that the new ligand has considerable amount of cross-reactivity with each of the receptors; $K_{3,1} = 1 \times 10^{11} / M$, $K_{3,2} = 1 \times 10^{10} / M$. Calculation of the fluorescent intensity of each bead in the presence of the third ligand yields the pattern in Fig. 18A which reveals an increase in the intensity of Bead 1 due to the third ligand, while leaving the intensity of Bead 2 unaffected due to the higher concentration of L_2 in the system and the lower affinity
 20 of L_3 to R_2 . Thus, L_3 may be detected under the condition that it has a relatively high affinity to one of the receptors and is in significant amount compared to the competing ligand.

The evaluation of the coaffinity matrix (and comparison with theoretical modeling as disclosed herein) under conditions in which a mixture of ligands is permitted to interact with a multiplicity of receptors arranged in a random encoded bead array format
 25 provides a methodology to establish a characteristic feature set of cross-correlations in the mutual competitive binding affinities of multiple ligands and receptors. These co-affinities provide a robust means to characterize receptor-ligand binding equilibria by their affinity fingerprinting patterns. Deviations from well-defined reference cases also permit detection of "perturbing" ligands in solutions.

Example 14. Multiplexed Analysis of Reaction Kinetics

As illustrated in the foregoing examples, extensive washing generally is not required to discriminate beads from a background of solution fluorescence. Consequently, assay image sequences may be recorded in a homogeneous assay format to document the evolution of a binding reaction and to determine kinetic data for each of the binding reactions occurring.

Homogeneous binding assays may be performed in simple “sandwich” fluidic cartridges permitting optical microscopic imaging of the bead array and permitting the introduction of an analyte solution into a chamber containing a random encoded array of beads. More generally, the array also may be exposed to an analyte or other reaction mixtures under conditions of controlled injection of fluid aliquots or continuous flow of reactants or buffer. Using theoretical modeling, optimal combinations of relevant performance control parameters of this bead array reactor may be identified to minimize the time to equilibration or to maximize the portion of analyte captured by the array [K. Podual and M. Seul, TM KP-99/02]. Flow rate can be controlled by any of a number of available pumping mechanisms [M. Freemantle, *C&EN*, 77: 27-36].

Table - List of parameters used in simulations (Fig. 18)

Parameter, units	Value
<i>Initial Receptor Coverage $c_{R,0}$, moles/m²</i>	8×10^{-9}
<i>Vol Flow Rate, Q, $\mu\text{l/s}$</i>	1.0
<i>Diffusivity, D, cm²/s</i>	1×10^{-7}
<i>ON-Rate, k_{on}, $1/(\text{M s})$</i>	1×10^5
<i>Affinity Constant, K_A, $1/\text{M}$</i>	1×10^{11}
<i>“Sandwich” Reactor Gap Size H, mm</i>	0.1
<i>Reactor Length, L, mm</i>	10
<i>Reactor Width, W, mm</i>	10

The analysis of image sequences permits kinetic data to be generated from which ON-rates and OFF-rates are determined with the aid of a theoretical model of the reaction-diffusion kinetics of the type illustrated in the foregoing example in Fig. 19. Fig. 19A displays stages in an adsorption-desorption cycle involving solution-borne analytes and a bead array immobilized at the bottom of a “sandwich” reaction chamber. The first panel depicts the initiation of the adsorption process; the second panel depicts the state of the reactor close to equilibrium when most of the beads have reached equilibrium; the last panel depicts the state of the reactor under the desorption cycle in which ligand-free fluid is injected and adsorbed molecules desorb from the bead surface. Fig. 19B displays the adsorption-desorption kinetics of a single receptor-single ligand system obtained by numerical solution of a reaction-diffusion system for a single type of receptor-ligand reaction; two cases of different concentrations of ligand are shown. Parameters used in the simulation are listed in the accompanying Table.

In contrast to prior art methods [D. G. Myszka, *Curr. Opin. Biotechnol.* 8: 50-57.], the present method relies on imaging and permits multiplexing. In addition, generalized models of the type introduced in Example 6 permit the analysis of complex binding kinetics for multiple simultaneous receptor-ligand interactions even in the presence of cross-reactions between multiple ligands and receptors.

The ability to monitor reaction kinetics in an array format will enable several approaches to enhancing the specificity of receptor-ligand or binding agent-analyte interactions in complex mixtures. For example, temperature programming may be invoked to enhance the specificity of DNA hybridization reactions. Similarly, the stringency of conditions applied to a hybridization reaction may be varied while the array response is being monitored; for example, hybridization may be conducted in a hybridization buffer under conditions leading to excess “non-specific” binding; specificity is enhanced by switching to a wash buffer of increasing stringency while monitoring the array response.

Example 15. Multi-Step Assay Sequences Using Encoded Arrays of Magnetic Particles

Methods and apparatus using biochemically functionalized super-paramagnetic particles for sample preparation in molecular and cellular biology and for a variety of enzyme-catalyzed on-bead reactions have been described ["Biomagnetic Techniques in Molecular Biology", Technical Handbook, 3rd Edition, 1998, Dynal, Oslo, NO]. These bead-based
5 methods can be combined with the Random Encoded Array Detection format of the present invention to implement multi-step on-chip assay manipulations.

For example, Fig. 20 illustrates the integration of a sequence of steps in a miniaturized format for multiplexed genotyping using a single chip with multiple compartments. First, cells are captured from a patient sample by affinity selection using
10 functionalized magnetic beads, cells are lysed electrically or chemically in a first compartment, and genomic DNA is captured to the surface of a multiplicity of magnetic beads by non-specific binding; next, beads are collected by magnetic force into a second compartment which is in fluidic contact with the first compartment, within which the beads and DNA are washed with desired buffers; next, beads are further transferred to a location where PCR is performed
15 using bead-coupled DNA as a template; multiple PCR strategies known in the art are available for this step [F. Fellmann, et.al., *Biotechniques*, 21:766-770]; next, PCR products released into are captured by hybridization to a pre-assembled random encoded array displaying binding agents that are specific to different polymorphisms targeted by the PCR amplification.

The use of encoded magnetic particles in conjunction with the optical
20 programmability of LEAPS confers the ability to form reversibly immobilized arrays and to conduct programmable multi-step assay sequences under conditions in which beads are used in suspension when this is most favorable, for example to enhance reaction kinetics, and arrays are formed in real-time when this is most favorable, for example to provide a highly parallel format for imaging detection and assay read-out.

25 For example, as illustrated in Fig. 21, the following sequence of steps could be integrated in a miniaturized format for the formation of a cDNA bead array. First, a pool of encoded magnetic beads, each bead type displaying a gene-specific probe, is introduced to an mRNA pool, and mRNA molecules are hybridized to their corresponding beads; next, on-bead reverse transcription (RT) is performed using bead-attached mRNA as template [E. Horenes,

L. Korsnes, US 005759820]; next mRNA is released from the beads; next beads are directed to the surface of a custom-designed chip and a cDNA bead array is formed using LEAPS. Such an array could serve to display binding agents in a gene profiling experiment using another set of mRNA as the target. Alternatively, the cDNA array could be analyzed for its own expression by applying a pool of labeled DNA binding agents to profile the genes of interest within the array.

Example 16. Synthesis of super-paramagnetic iron oxide γ -Fe₂O₃ (maghemite) particles

The synthesis was carried out in reversed micellar solutions composed of the anionic surfactant, bis(2-ethylhexyl)sodium sulfosuccinate (AOT) and isooctane (Kommareddi et al., Chem. Mater. 1996, 8, 801-809) obtained from Aldrich Chemical Co., Milwaukee, WI. Stock solutions of 0.5M AOT were used in preparing the reversed micellar solutions containing the reactants FeSO₄ (Sigma Chemical Co., St. Louis, MO) and NH₄OH (Sigma Chemical Co., St. Louis, MO). Specifically, 0.45ml of 0.9M FeSO₄ was added to 5 ml of 0.5M AOT in isooctane, separately 0.45ml of NH₄OH was added to 5 ml of 0.5M AOT in isooctane. The reaction was initiated by adding the NH₄OH reversed micellar solution to the FeSO₄ reversed micellar solution under vigorous stirring. The reaction was allowed to proceed for ~2-3hrs and then the solvent was evaporated at ~ 40°C to obtain a dry surfactant iron oxide composite. This composite was re-dispersed in the organic solvent of choice to give a deep red colored transparent solution.

Example 17. Synthesis of fluorescently colored and magnetic polymer bead composites

A stock solution of hydrophobic fluorescent dye and the iron oxide particles was made by re-dispersing the dried magnetic composite and the dye in the solvent of choice, for example

a CHCl_3 (Aldrich Chemical Co., Milwaukee, WI) or $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ mixture (70/30 (v/v)) (Aldrich Chemical Co., Milwaukee, WI). A predetermined amount of polymer beads was washed thoroughly in methanol (3x) and then evaporated dry. Simultaneous incorporation of the fluorescent dye and the iron oxide nanoparticle was achieved by swelling the beads in
5 organic solvent/nanoparticle/dye mixture. The swelling process was completed within ~ 1hr. Following this the polymer beads were separated by centrifugation and washed with methanol(3x) followed by isooctane(2x) and then methanol(2x) and finally redispersed in 0.2% SDS –DI water solution.

10

WHAT IS CLAIMED IS :

1. A method of determining elements of a co-affinity matrix which describes pair-wise
5 analyte-binding interactions in a competitive multiconstituent equilibrium reaction comprising:
providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely
10 identifies the binding agents on said particles, and wherein the particles are arranged in a planar array on a substrate;
determining the identity of said binding agents on each particle in the array by the chemically or physically distinguishable characteristic associated therewith;
contacting the binding agents with an analyte molecule so as to allow the analyte to
15 form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;
detecting the change in the optical signature associated with said particles for each type of the analyte-binding agent complexes formed; and
20 determining the identity of the binding agents involved in the complex formation by the chemically or physically distinguishable characteristic associated with the corresponding particles,
wherein said change in optical signature for each type of the analyte-binding agent complexes determines affinities characterizing said analyte-binding agent interaction and said
25 affinities providing elements of a co-affinity matrix which describes pairwise analyte-binding agent interactions in a competitive multiconstituent equilibrium reaction.

2. A method of determining elements of a co-affinity matrix which describes pair-wise analyte-binding interactions in a competitive multiconstituent equilibrium reaction comprising:
- providing a plurality of particles comprising at least two different particle populations,
- 5 each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles,
- contacting the binding agents with an analyte molecule so as to allow the analyte to form an analyte-binding agent complex with one or more binding agents, the formation of each
- 10 complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;
- forming a planar array of the particles on a substrate;
- detecting the change in the optical signature associated with said particles for each type of the analyte-binding agent complexes formed; and
- 15 determining the identity of the binding agents on each particle in the array by the chemically or physically distinguishable characteristic associated therewith, the determining step occurring either before or after the detecting step, and
- wherein said change in optical signature for each type of the analyte-binding agent complexes determines affinities characterizing said analyte-binding agent interaction and said
- 20 affinities providing elements of a co-affinity matrix which describes pairwise analyte-binding agent interactions in a competitive multiconstituent equilibrium reaction.
3. The method of claim 1 or 2, wherein the co-affinity matrix obtained is used to characterize the analyte.
- 25
4. The method of claim 3, wherein a combination of the coefficients of the co-affinity matrix and known concentrations of analyte and binding agents participating in the formation of analyte-binding agent complexes serves to define a competitive binding interaction descriptor .

5. The method of claim 1 or 2, further comprising the determination of the affinity constant for each type of the analyte-binding agent interaction, wherein each affinity constant is calculated by determining the number of analyte-binding agent complexes formed from said change in the optical signature of each type of the analyte-binding agent complexes formed.
6. A method of determining the presence or absence of an impurity in a sample comprising:
- 10 preparing a co-affinity matrix of a reference analyte with a set of binding agents according to the method of claim 1 or 2, and recording said co-affinity matrix, introducing a sample containing said reference analyte and possibly an impurity analyte, and determining the co-affinity matrix subsequent to the addition of the sample; and detecting for the deviation in the coefficients of the co-affinity matrix of the reference
- 15 analyte subsequent to the addition of the sample that might contain an impurity, wherein the deviation in the co-affinity matrix indicates the presence of an impurity in said sample.
7. A method of analyzing the kinetics of molecular binding interactions governing the association of analyte-binding agent complexes formed between one or more analytes and one or more binding agents, comprising:
- 20 providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles, and wherein the particles are arranged in a planar
- 25 array on a substrate;
- determining the identity of said binding agents on each particle in the array by the chemically or physically distinguishable characteristic associated therewith;
- contacting, at a preset initial time, the binding agents in the array with an analyte molecule so as to allow the analyte to form an analyte-binding agent complex with one or more

binding agents, the formation of each complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;

detecting the change in the optical signature associated with said particles for
each type of the analyte-binding agent complexes formed at preset intervals following said
5 preset initial time; and

determining, for each type of analyte-binding agent complex, the association rate constants from the time dependence of said changes in optical signature associated with said particles.

10 8. A method of analyzing the kinetics of molecular binding interactions governing the dissociation of analyte-binding agent complexes formed between one or more analytes and one or more binding agents, comprising:

providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles
15 are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles, and wherein the particles are arranged in a planar array on a substrate;

determining the identity of said binding agents on each particle in the array by the chemically or physically distinguishable characteristic associated therewith;

20 contacting the binding agents in the array with an analyte molecule so as to allow the analyte to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;

detecting the change in the optical signature associated with said particles for
25 each type of the analyte-binding agent complexes formed;

exchanging, at a preset exchange time, the analyte solution with a second solution containing no analyte to allow the bound analytes to unbind from the analyte-binding agent complexes, said unbinding resulting in a change in the optical signature associated with the particles having analyte-binding agent complexes, and

recording, at preset intervals following said preset exchange time, said changes in optical signature and determining, for each type of analyte-binding agent complex, the dis-association rate constants from the time dependence of said changes in optical signature associated with said particles.

5

9. The method of any of claims 1 to 8, wherein the change in the optical signature comprises a change in the fluorescent intensity associated with the particles involved in the binding interaction.

10 10. The method of any one of claims 1 to 8, wherein the determining of the identity of the binding agents in the array comprises taking a decoding image of the array that detects the chemically or physically distinguishable characteristic of each bead in the array, and wherein the optical signature detecting step comprises taking an assay image of the array that detects the change in the optical signature associated with the beads.

15

11. The method of claim 10, wherein the assay image and the decoding image are compared using a template matching algorithm.

12. The method of any one of claims 1 to 8, wherein the planar particle array is immobilized
20 on the substrate.

13. The method of any one of claims 1 to 8, wherein the particles are associated with a chemically distinguishable characteristic.

25 14. The method of claim 13, wherein the chemically distinguishable tag comprises a fluorophore tag.

15. A method of performing a bioassay comprising:

- providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles, and wherein the particles are arranged in a planar array on a substrate;
- 5 generating a de-coding image of the array showing the location of each binding agent in the array;
- contacting the binding agents with a sample that may contain an analyte so as to allow the analyte, if present in the sample, to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a corresponding or a proportional change in the optical signature associated with the particles whose binding agent is involved in the formation of the complex;
- 10 generating an assay image of the array which detects the change in the optical signature associated with said particles, and deriving from the change in the optical signature the number of analyte-binding agent complexes formed; and
- 15 determining the identity of the analyte in the analyte-binding agent complex by comparing the decoding image with the assay image.

16. A method of performing a bioassay comprising:
- 20 providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles,
- contacting the binding agents with a sample that may contain an analyte so as to allow the analyte, if present in the sample, to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a corresponding or proportional change in the optical signature associated with the particles whose binding agent is involved in the formation of the complex;
- 25 forming a planar array of the particles on a substrate;

generating an assay image of the array which detects the change in the optical signature associated with said particles, and deriving from the change in the optical signature the number of analyte-binding agent complexes formed;

generating a decoding image of the array showing the location of each binding agent in
5 the array, wherein the decoding image is generated either before or after generating the assay image; and

determining the identity of the analyte in the analyte-binding agent complex formed by comparing the decoding image with the assay image.

10 17. The method of claim 15 or 16, wherein the comparing of the assay and the decoding image comprises use of optical microscopy apparatus including an imaging detector and computerized image capture and analysis apparatus.

15 18. The method of claim 15 or 16, wherein the analyte comprises a ligand and the binding agents comprise receptors.

19. The method of claim 15 or 16, wherein the analyte and the binding agents comprise nucleotide fragments, and wherein the number of hybridization complexes formed between the nucleotide fragment and its complementary sequence fragments is
20 determined.

20. The method of claim 15 or 16, wherein the particles comprise magnetic particles distinguishable by a chemical or physical characteristic that uniquely identifies the binding agent attached thereto.

25 21. The method of claim 20, wherein the chemical or physical characteristic comprises one or more fluorophore dyes spectrally distinguishable by excitation wavelength, emission wavelength, excited-lifetime or emission intensity.

22. The method of claim 21, wherein the array of magnetic particles is assembled by application of a magnetic field to said particles.
23. The method of claim 15 or 16, further comprising calculating the affinity constant of a
5 binding interaction between an analyte and its binding agent from the number of the analyte-binding agent complexes formed.
24. A method of integrating sample preparation and bioassay using magnetic particles comprising:
10 providing a plurality of magnetic particles comprising at least two different particle populations, each population being distinguishable by a recognition molecule attached thereto, wherein the particles are attached to a chemical characteristic that uniquely identifies a biomolecule of interest that selectively binds to the recognition molecule;
providing a biological fluid containing biomolecules and allowing said biomolecules to
15 interact with the recognition molecules on the magnetic particles;
removing the fluid along with unbound components thereof;
transforming the biomolecules bound to the magnetic particles to produce transformed biomolecules, wherein the transformed biomolecules remain attached to the magnetic particles on which they are synthesized;
20 performing a bioassay according to the method of claim 15 or 16, wherein the binding agents comprise the transformed biomolecules.
25. The method of claim 24, wherein the biomolecules of interest comprises mRNA and the transforming comprises reverse transcribing said mRNA to produce cDNA, which is
25 attached to the magnetic particles.
26. The method of claim 24, wherein the sample preparation and bioassay occur in the same compartment.

27. A method for performing a bioassay involving integration of sample preparation and parallel molecular interaction assay analysis, comprising

providing an apparatus comprising at least a sample preparation compartment and an assay compartment, and means for fluidically connecting the sample and the assay compartments;

providing, in the sample preparation compartment, a biological fluid containing a biomolecule of interest and a plurality of magnetic particles capable of binding to the biomolecule of interest, and allowing the magnetic particles to bind the biomolecules of interest;

removing the biological fluid along with unbound components of said fluid, while retaining the magnetic particles and the biomolecules bound to said particles;

releasing said biomolecules from said magnetic particles and transporting said biomolecules from the sample preparation compartment to the assay compartment through the fluidic means; and

performing a bioassay according to the method of 15 or 16, wherein the analyte in the bioassay comprises transported biomolecules of interest.

28. The method of claim 27, further comprising transforming the biomolecule of interest, which is then used as an analyte in the bioassay.

29. The method of claim 27, wherein the biomolecule of interest comprises mRNA and the transformation comprises reverse transcription of said mRNA to produce cDNA, and wherein the analyte in the bioassay comprises said cDNA and the binding agents comprises oligonucleotides or other DNA probes.

30. The method of claim 29, wherein the reverse transcription occurs in the sample preparation compartment, while said mRNA is bound to the magnetic particles, and the

cDNA is released from said magnetic particles after the reverse transcription and transported to the assay compartment and used as an analyte in the bioassay.

31. A method of preparing monodisperse magnetic fluorescent particles comprising
5 providing polymeric microparticles and swelling said microparticles in an organic solvent containing one or more and magnetic nanoparticles to produce magnetic particles, wherein the fluorescent dyes and the magnetic nanoparticles are distributed throughout the magnetic particles without being localized at specific locations with the particle.
- 10 32. An array comprising a plurality of magnetic particles comprising at least two different particle populations distinguishable by a chemical characteristic associated therewith, wherein the magnetic particles are assembled on a planar array in a compositionally random manner.
- 15 33. The method of claim 5, wherein the analyte specifically binds to one binding agent but not to other binding agent(s) present.
34. The method of claim 33, wherein, using the law of mass action, affinity constant K is determined from said change in optical signature following conversion to yield
20 $[LR]$, given $[L_o]$, $[R_o]$, and n_B , where $[L_o]$ is the initial analyte concentration, $[R_o]$ is the number of binding agents per bead and n_B is the number of beads.
35. The method of claim 33, wherein, using the law of mass action, affinity constant K is determined from said change in optical signature following conversion to yield
25 $[LR]$, for at least two measurements involving different bead numbers, n_B and given initial analyte concentration $[L_o]$.
36. The method of claim 33, wherein, using the law of mass action, affinity constant K is determined from said change in optical signature following conversion to yield

[LR], for at least two measurements involving different number of binding agents per bead, $[R_o]$ and given initial analyte concentration $[L_o]$.

37. The method of claim 33, wherein, using the law of mass action, concentration
5 of analyte $[L]$, is determined from said change in optical signature following conversion to yield $[LR]$, given K , n_B and $[R_o]$

38. The method of claim 33, wherein in a two-step assay involving the use of a first
10 labeled analyte of interest in the first binding reaction, followed by injection of a second analyte with known affinity constant K' , the affinity constant K is determined from said the optical signature before and after injection of the second analyte, following conversion to yield $[LR]_1$ and $[LR]_2$ given the initial concentrations of the analytes, $[L_o]$ and $[L_o]'$ and $[R_o]$.

15 39. The method of claim 33, wherein
in a two-step assay involving the use of a first labeled analyte of interest in the first binding reaction, followed by injection of a second analyte with known affinity constant K' , the affinity constant K is determined from said the optical signature before and after injection of the second analyte, following conversion to yield $[LR]_1$ and $[LR]_2$ given the initial
20 concentrations of the analytes, $[L_o]$ and $[L_o]'$ and n_B .

40. The method of claim 33, wherein:
using the law of mass action, the initial analyte concentration $[L_o]$ is determined from said
change in optical signature following conversion to yield $[LR]$, given K , $[R_o]$, and n_B .
25

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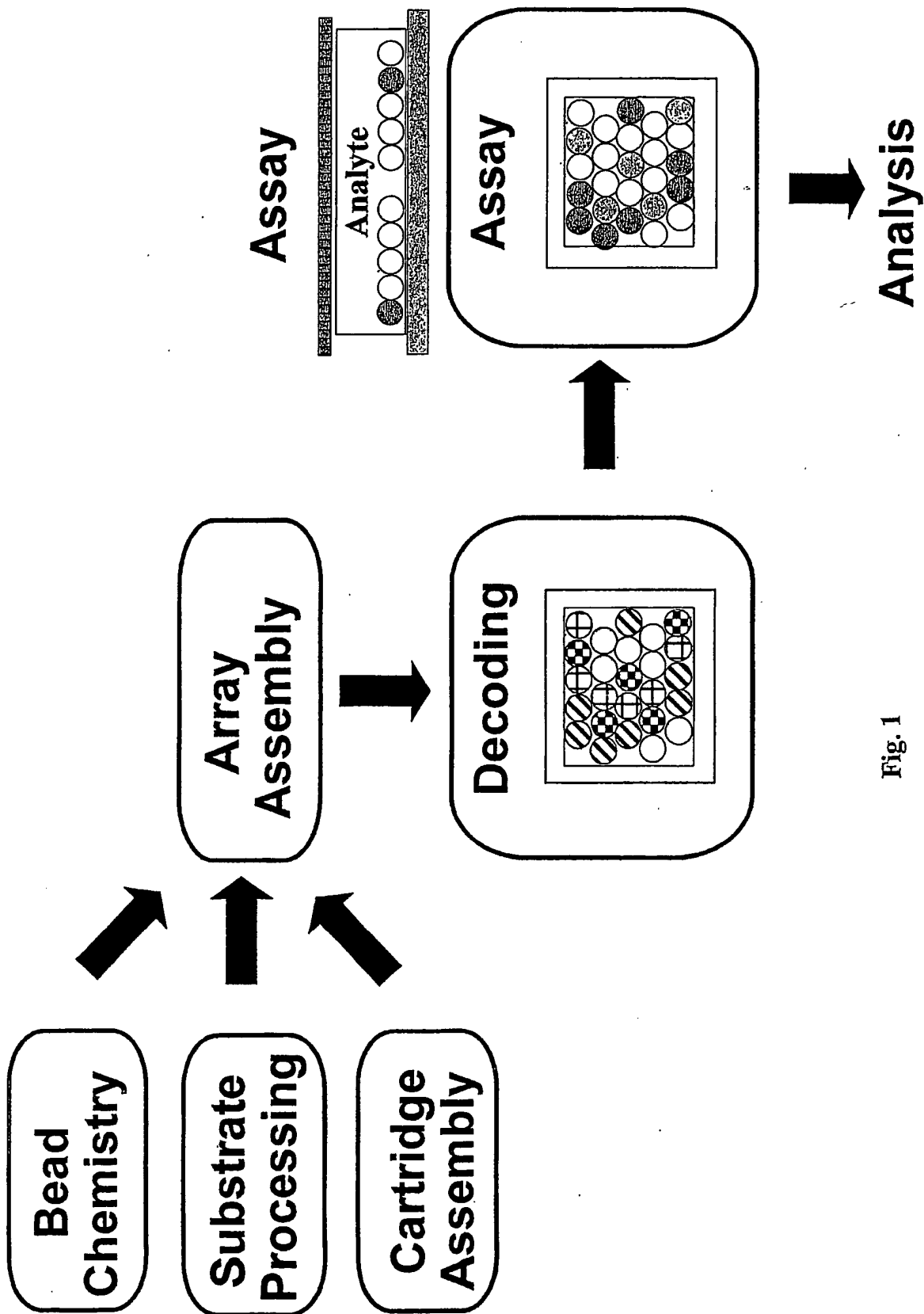


Fig. 1

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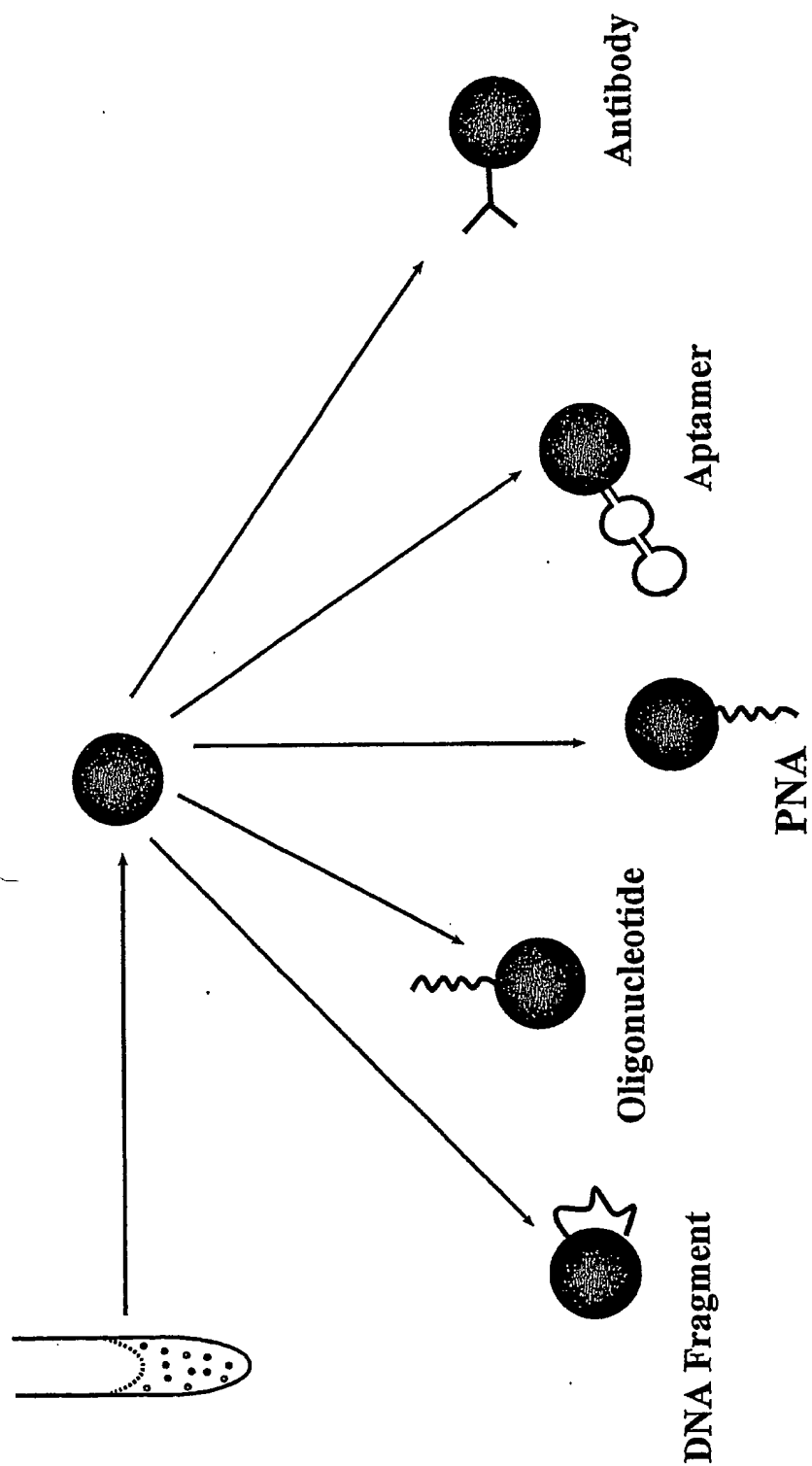


Fig. 2

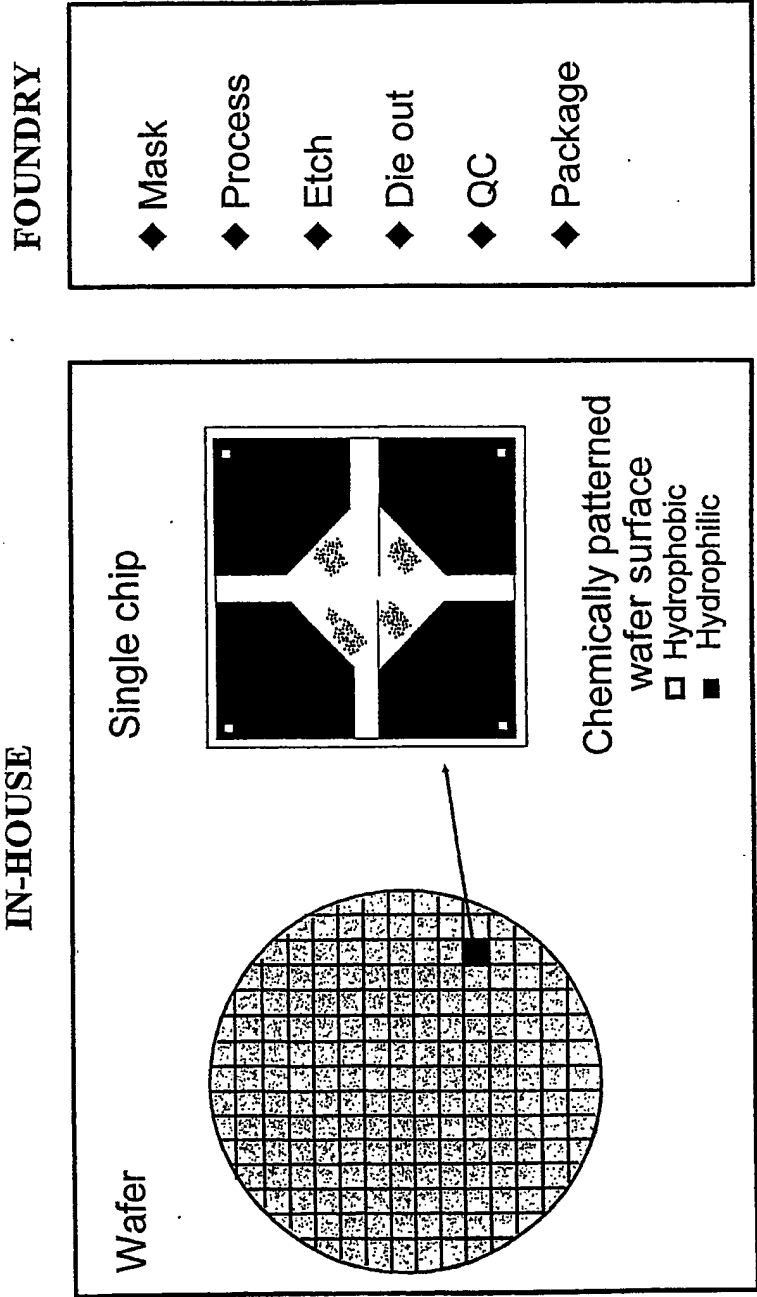


Fig. 3

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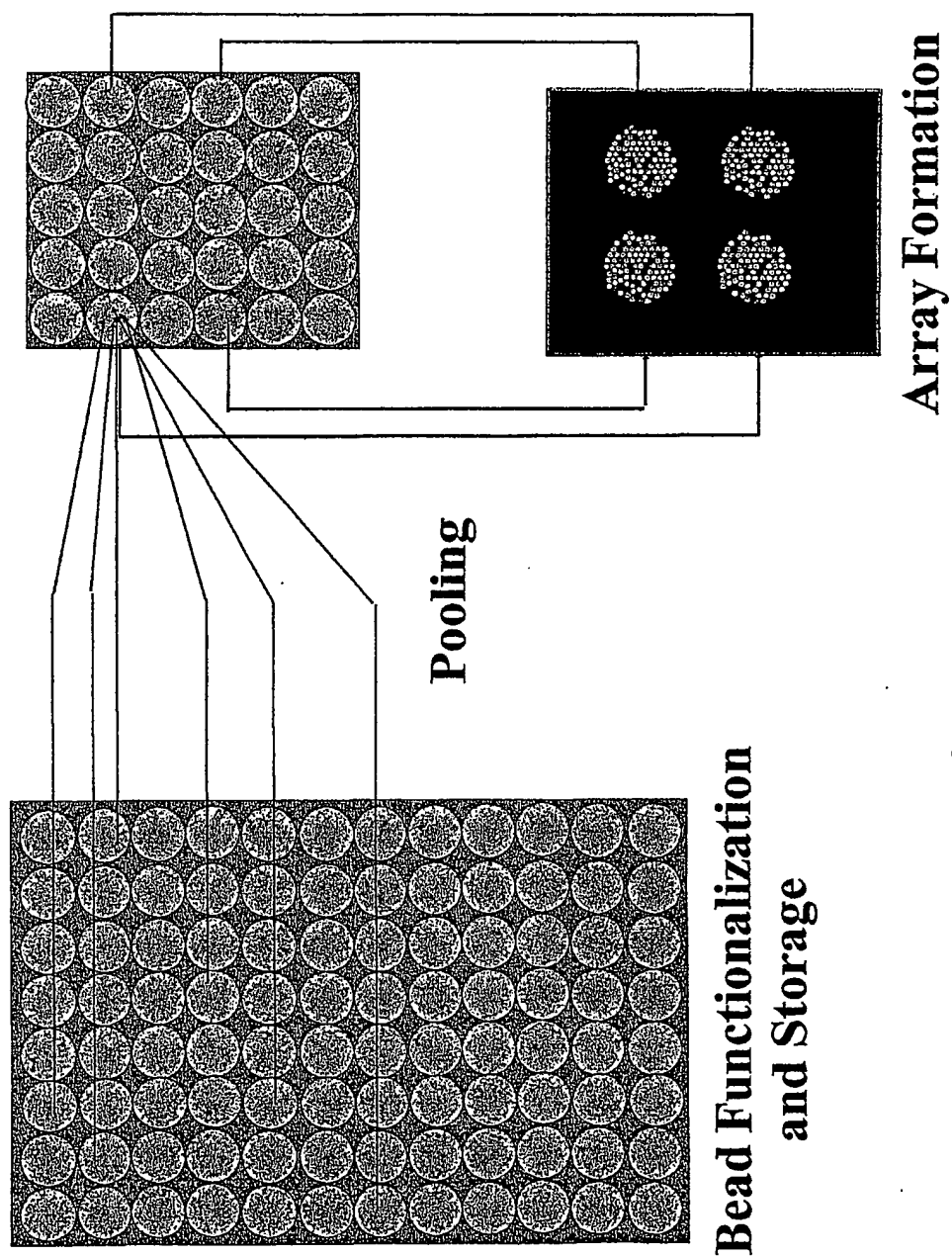


Fig. 4

5/22

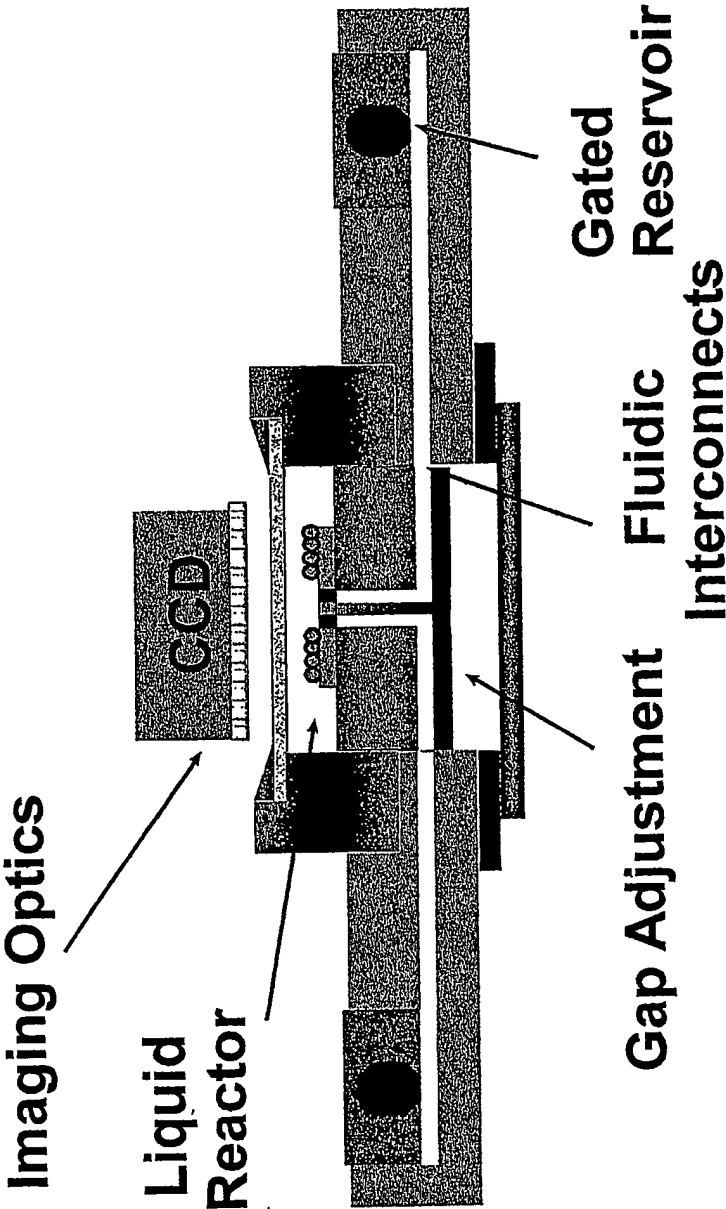


Fig. 5

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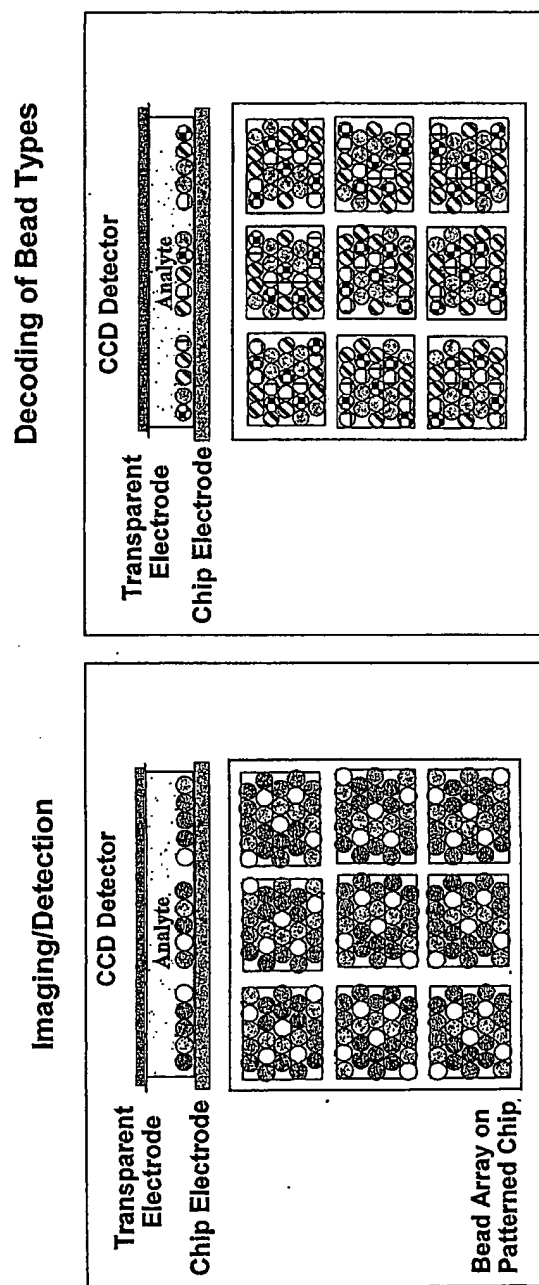


Fig. 6

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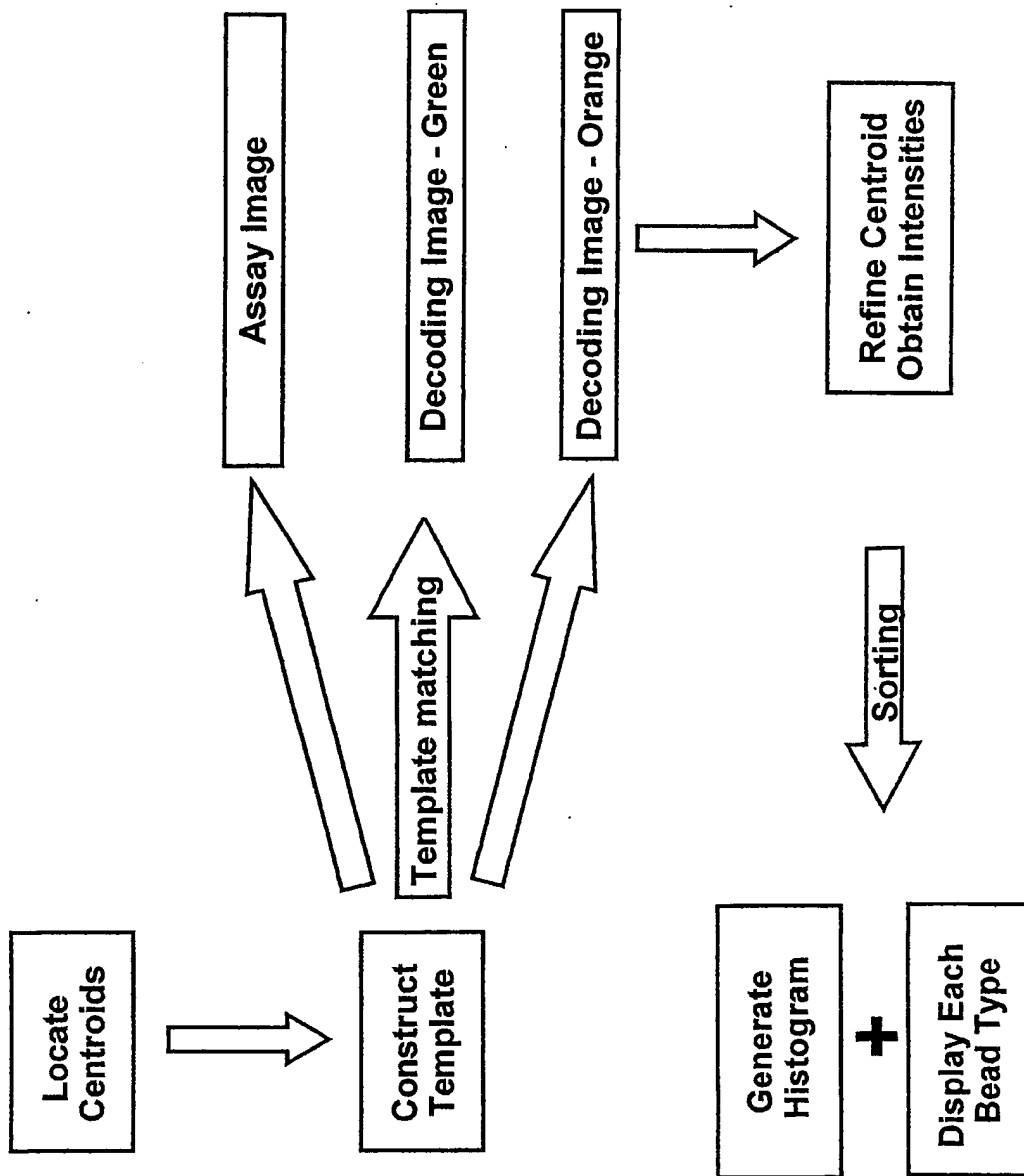


Fig. 7

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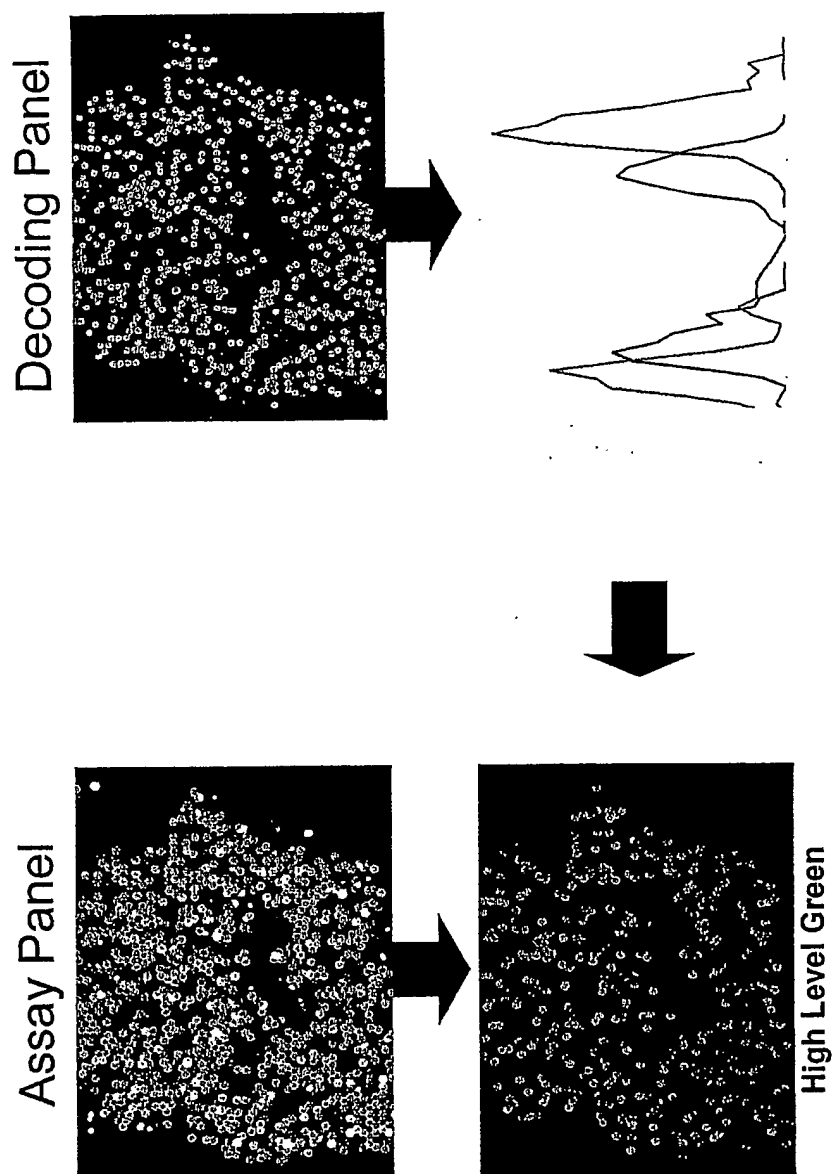


Fig. 8

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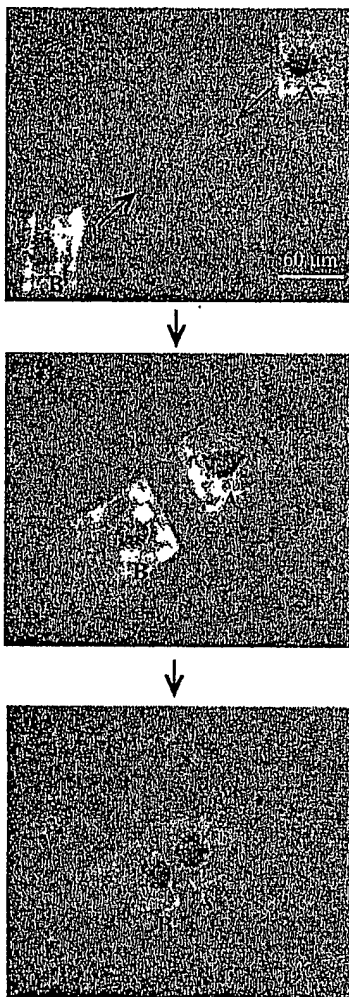


Fig. 9

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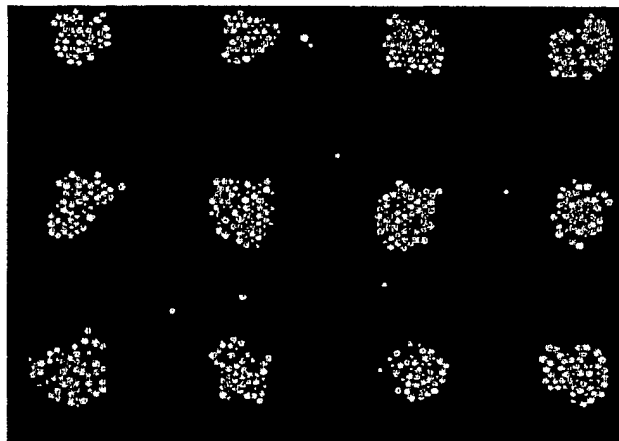


Fig. 10

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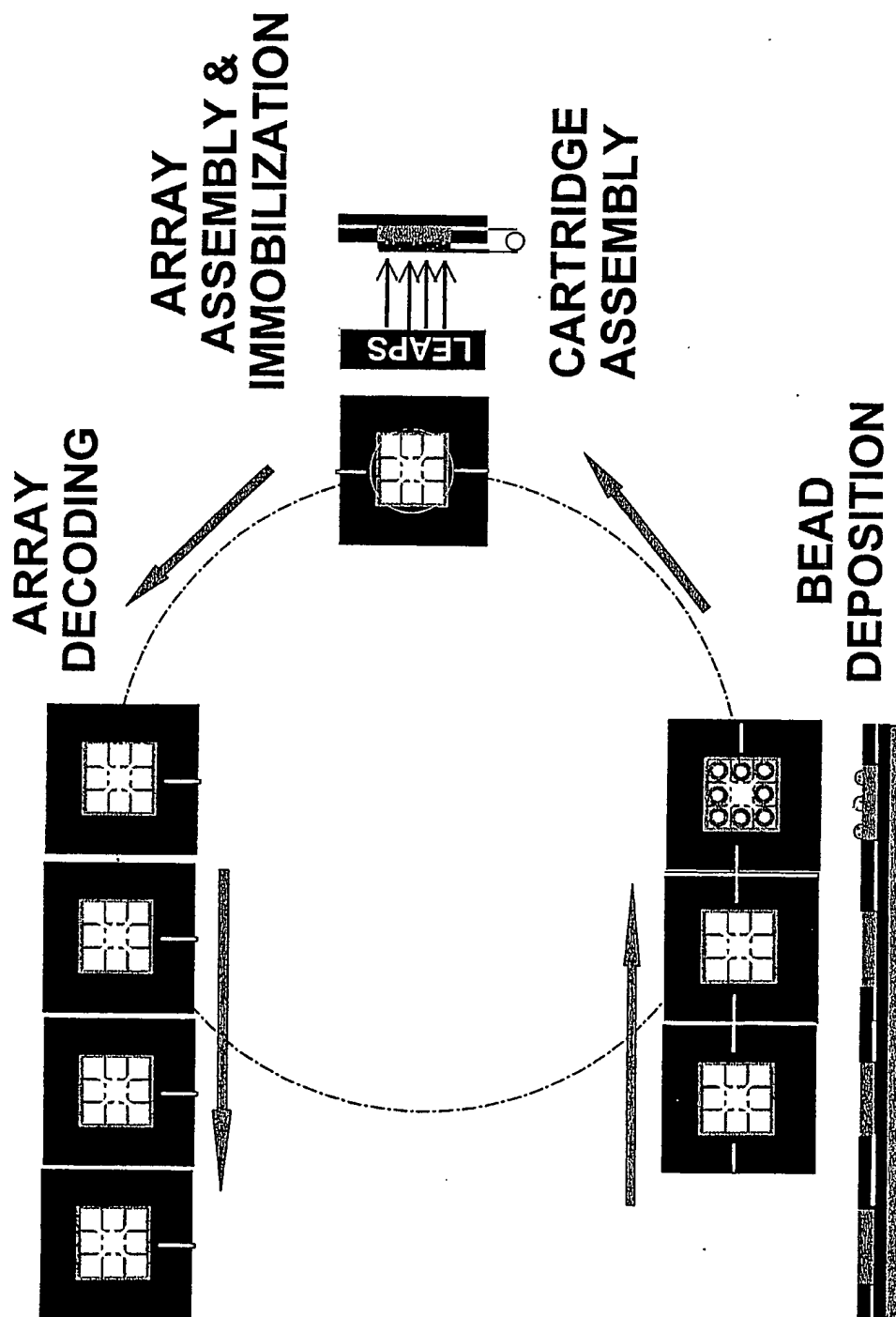


Fig. 11

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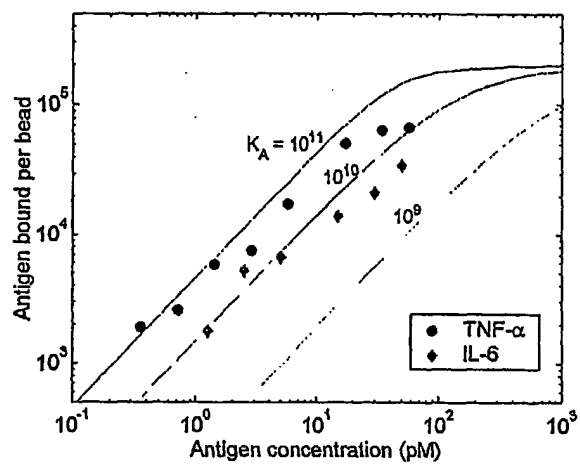


Fig. 12

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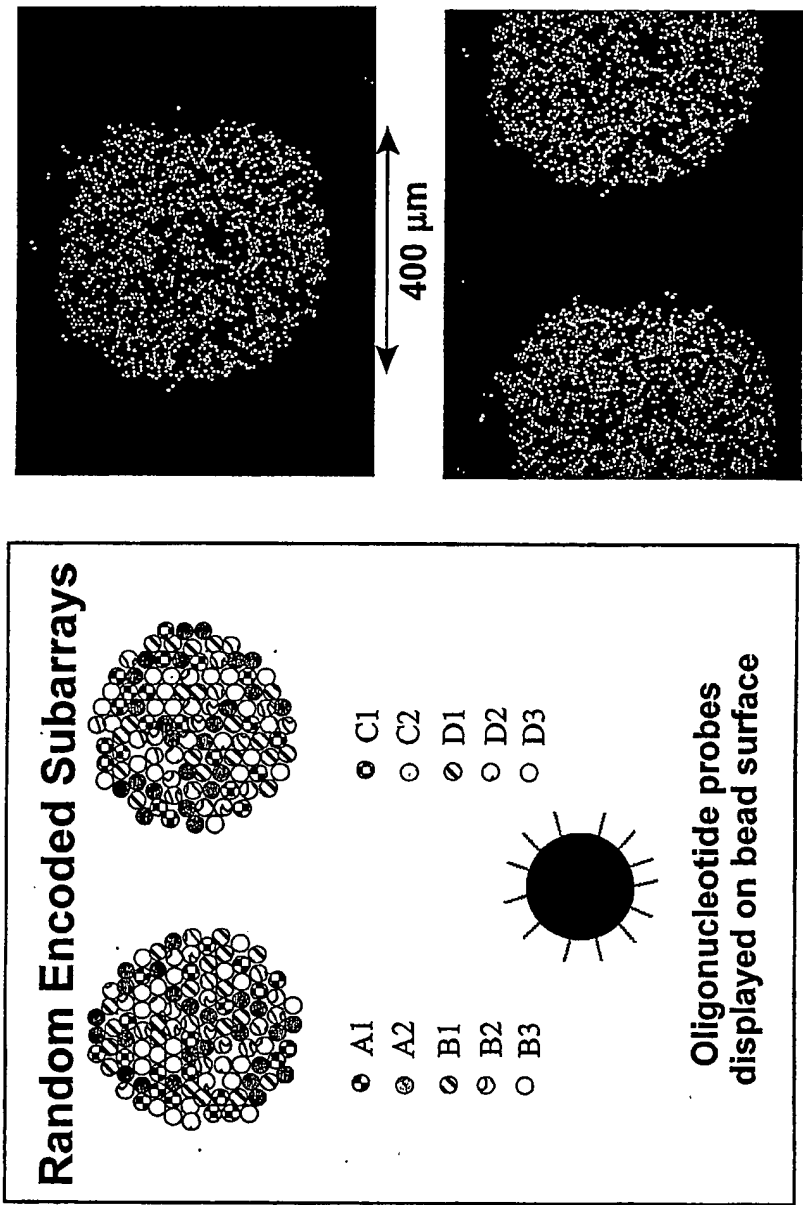


Fig. 13

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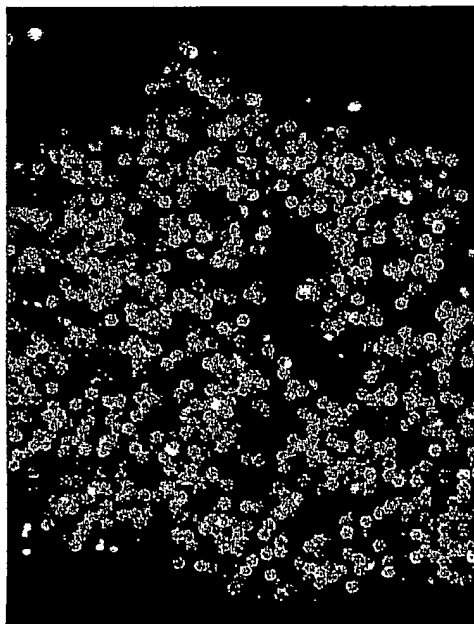
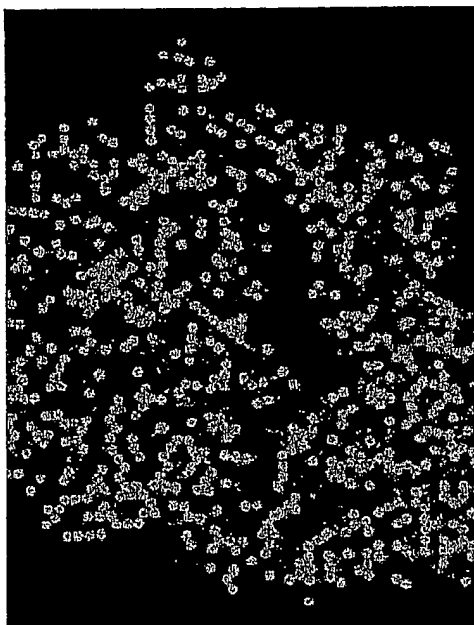


Fig. 14

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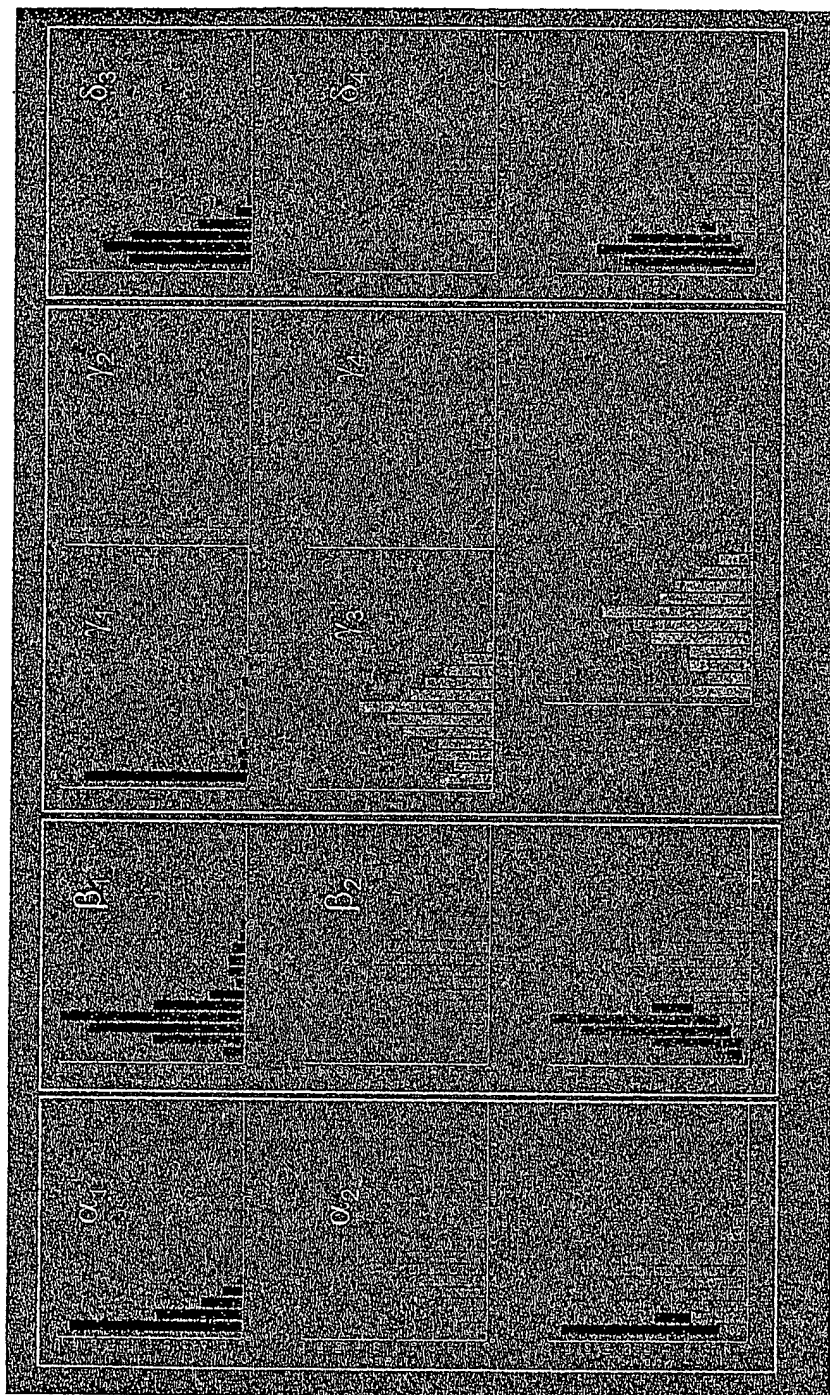


Fig. 15

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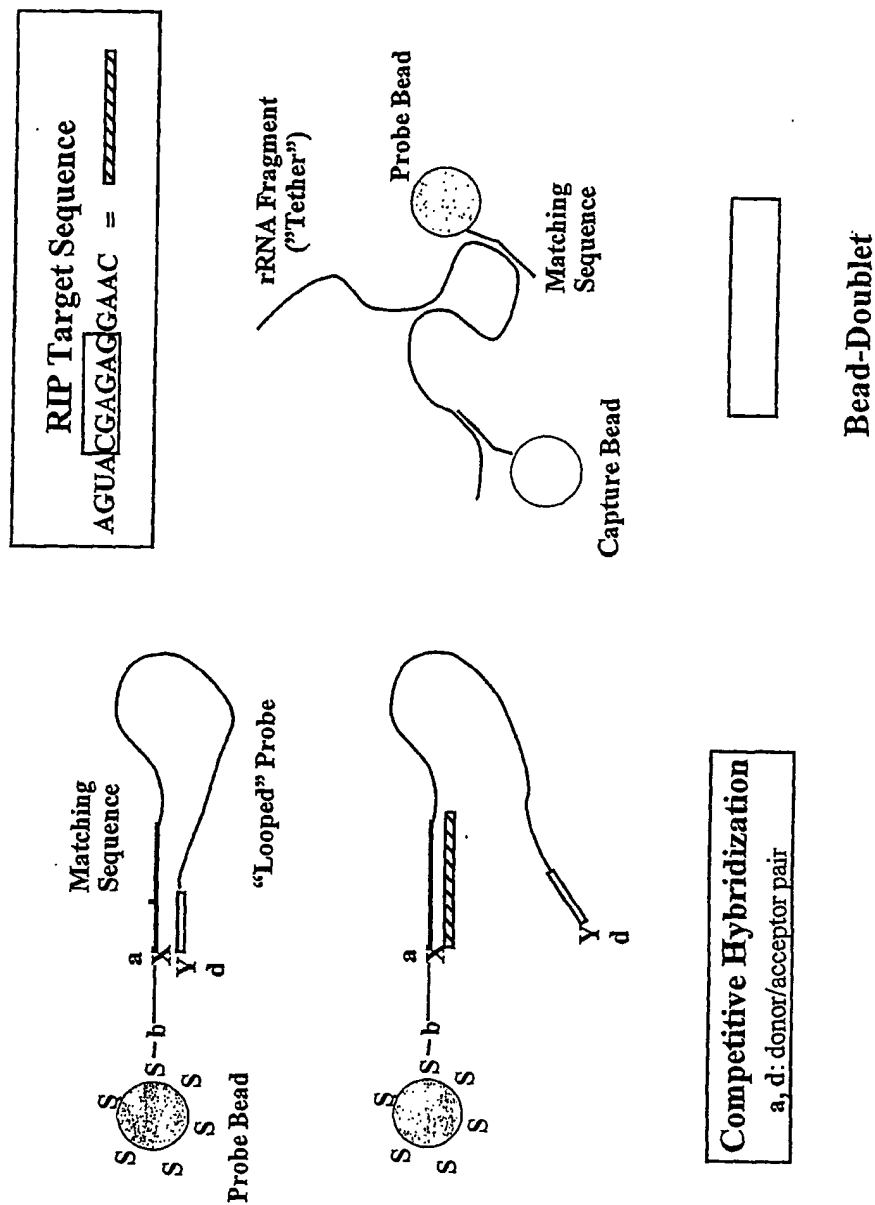


Fig. 16

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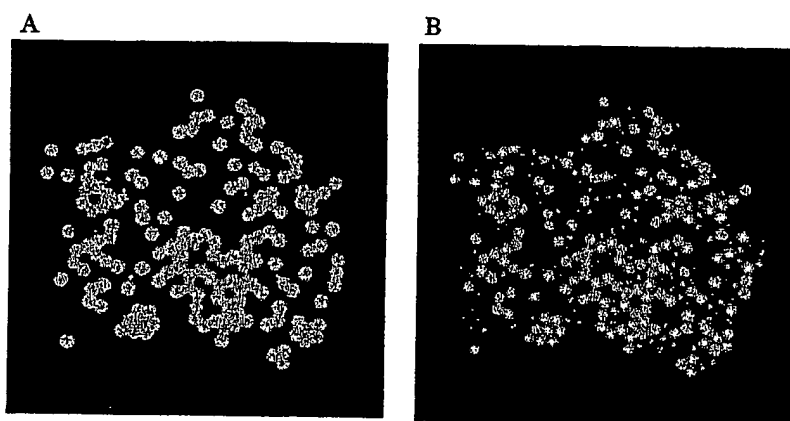


Fig. 17

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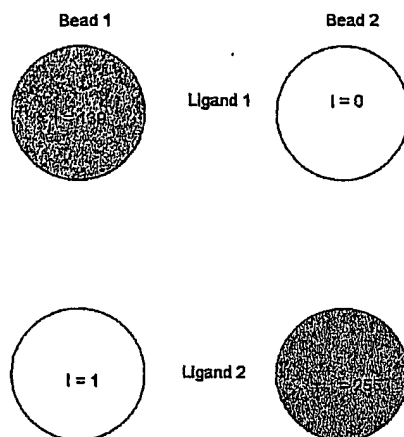


Fig. 18A

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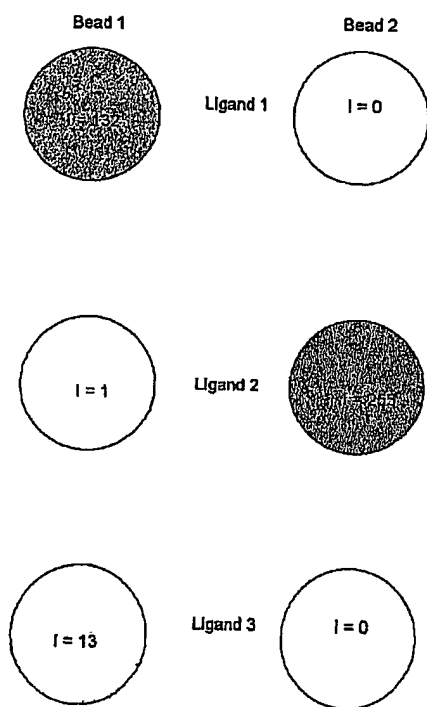


Fig. 18B

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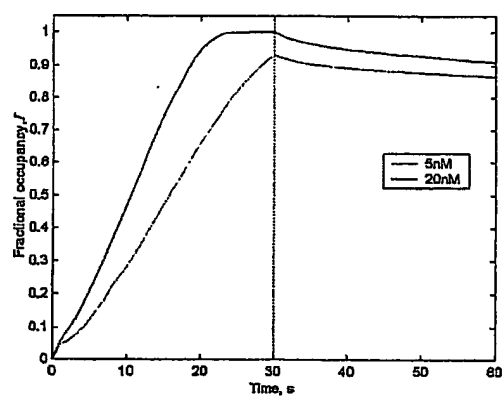
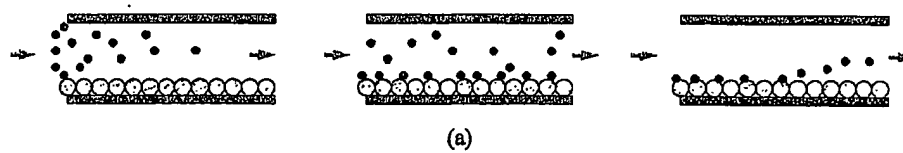


Fig. 19

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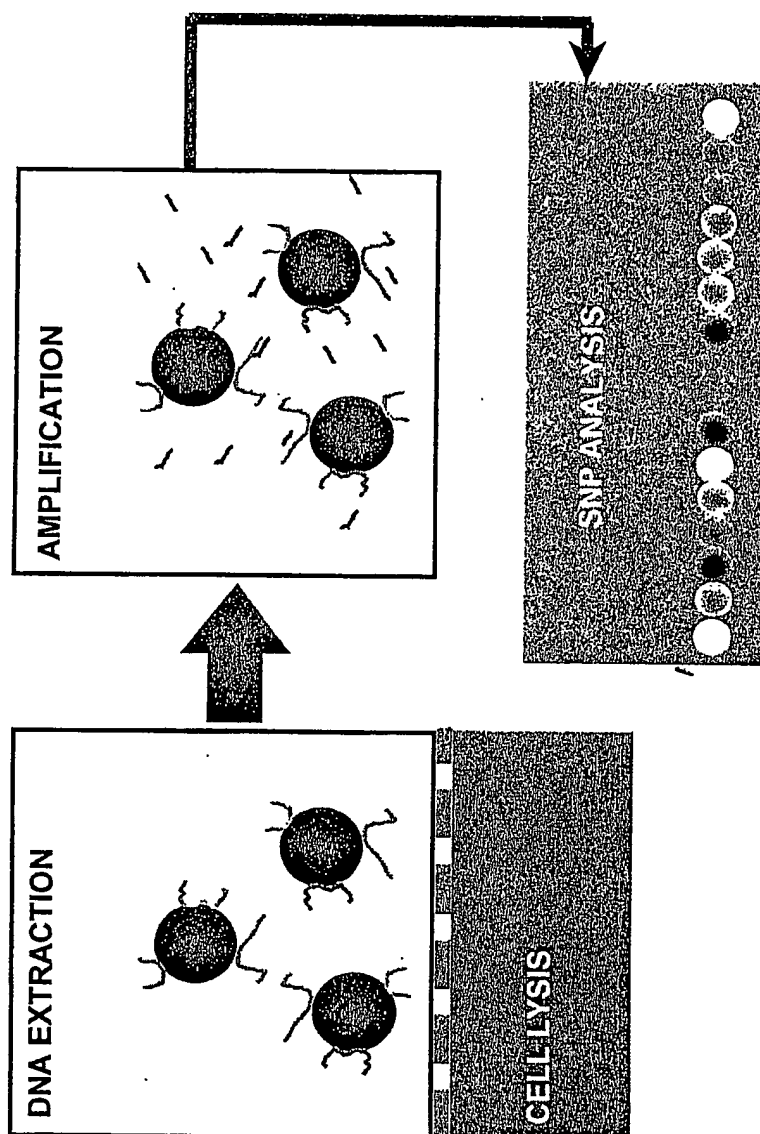


Fig. 20

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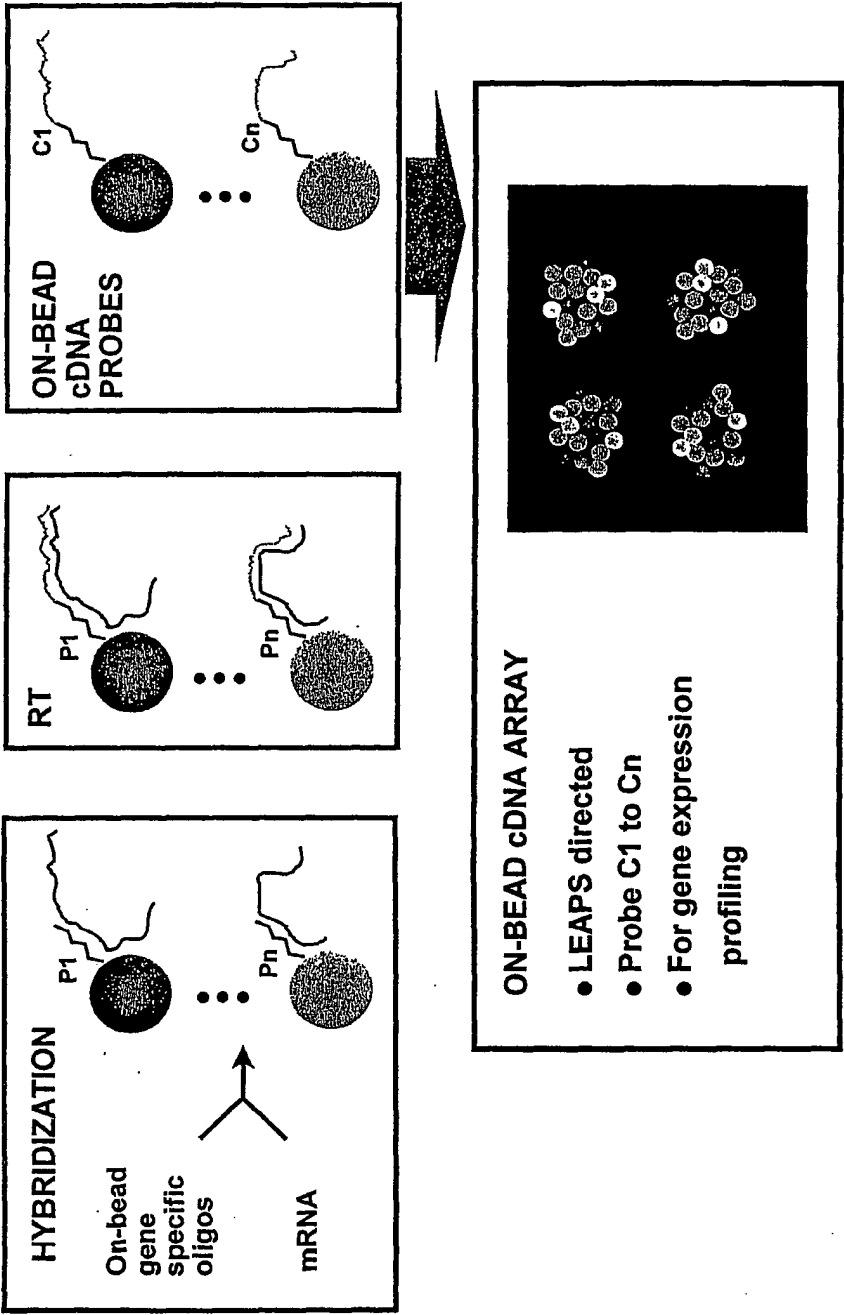


Fig. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/20179

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : G01N 27/26, 33/553, 21/00; C12P 19/34 US CL : Please See Extra Sheet. According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : Please See Extra Sheet. Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PALM, EAST, WEST														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
A,E	US 6,251,691 B1 (SEUL) 26 June 2001, see col. 5, line 55-col. 7, line 9.	1-8, 15-40												
A, E	US 6,268,222 B1 (CHANDLER et al.) 31 July 2001, see col. 2, line 60-col. 5, line 42.	1-8, 15-40												
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier document published on or after the international filing date</td> <td>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Z" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier document published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention													
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
"E" earlier document published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family													
"O" document referring to an oral disclosure, use, exhibition or other means														
"P" document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 01 OCTOBER 2001		Date of mailing of the international search report 16 NOV 2001												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer FENSEE T. DO Telephone No. (703) 305-0196												

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/20179

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 9-14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/20179

A. CLASSIFICATION OF SUBJECT MATTER: US CL :

436/534, 501, 518, 524, 526, 528, 531, 169, 172, 175; 422/50, 54, 55, 56, 57, 58, 68.1, 69, 82.01-82.03, 82.05, 129, 131, 138, 186.04; 435/91.2, 91.5, 177, 178.1, 174, 176, 283.1; 204/416, 417

B. FIELDS SEARCHED Minimum documentation searched Classification System: U.S.

436/534, 501, 518, 524, 526, 528, 531, 169, 172, 175; 422/50, 54, 55, 56, 57, 58, 68.1, 69, 82.01-82.03, 82.05, 129, 131, 138, 186.04; 435/91.2, 91.5, 177, 178.1, 174, 176, 283.1; 204/416, 417